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C3H
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(54) Genetic engineering

(57) It has been a problem to find an alternative, less time-consuming, and more reliable source of factor IX, a polypeptide which is essential to the human blood-clotting process and necessary for the treatment of patients with Christmas disease. In order to aid in the solution of the problem, there is provided recombinant DNA containing a DNA sequence occurring in the human factor IX genome, and includes recombinant DNA comprising substantially the whole sequence of human factor IX genome, which is inserted in a cloning vehicle and transformed into a host, such as *Escherichia coli*. Other fragments of the sequence have also been cloned and the invention includes DNA molecules comprising part or all of the human factor IX DNA. There is also described cDNA derived from human factor IX RNA. Uses include the provision of an intermediate of value in the genetic engineering of a factor IX polypeptide precursor and thence manufacture of the factor IX polypeptide, and in making probes for use in diagnosing the presence of normal or abnormal factor IX DNA in patients with Christmas disease.

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1st amino acid sequence : 70 Glu-Cys-Trp-Cys-Gln-Ala 75

mRNA : 5' GA$^{A}_{G}$ UG$^{U}_{C}$ UGG UG$^{U}_{C}$ CA$^{A}_{G}$ GCN 3'

Deoxyoligonucleotides synthesized :

3' CT$^{T}_{C}$ AC$^{A}_{G}$ ACC AC$^{A}_{G}$ GTT CG (oligo N2A)

3' CT$^{T}_{C}$ AC$^{A}_{G}$ ACC AC$^{A}_{G}$ GTC CG (oligo N2B)

2nd amino acid sequence : 348 His-Met-Phe-Cys-Ala 352

mRNA : 5' CA$^{U}_{C}$ AUG UU$^{U}_{C}$ UG$^{U}_{C}$ GCN 3'

Deoxyoligonucleotides synthesized : GT$^{A}_{G}$ TAC AA$^{A}_{G}$ AC$^{A}_{G}$ CG (oligo N1)

Fig. 1

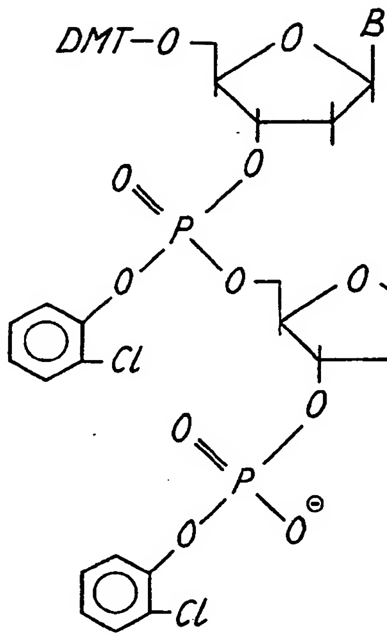

Fig. 2

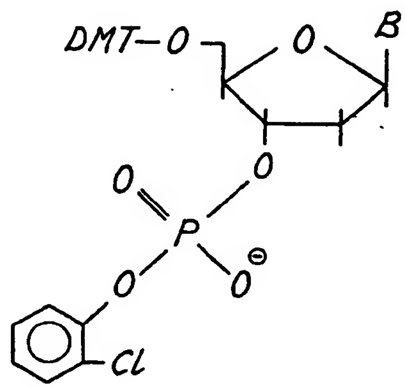

Fig. 3

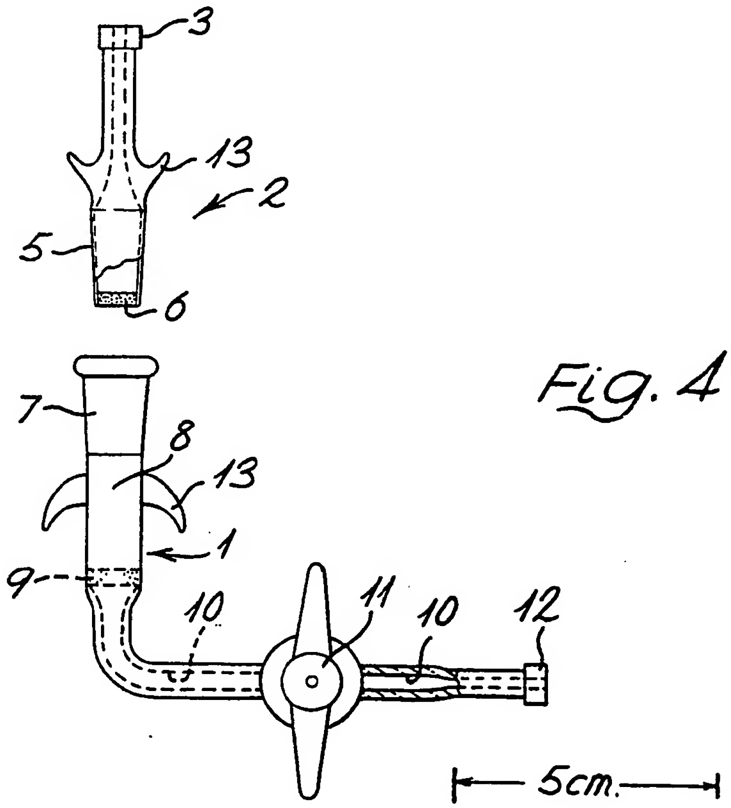

Fig. 4

```
                     60
  E  S  N  P  C  L  N  G  G  M  C  K  D  D  I  N  S  Y
5' TGAATCCAATCCATGTTTAAATGGCGGCATGTGCAAGGATGACATTAATTCCTAT
         10        20        30        40        50

70                       80                       90
E  C  W  C  Q  A  G  F  E  G  T  N  C  E  L  D  A  T  C  S  I  K
GAATGTTGGTGTCAAGCTGGATTTGAAGGAACGAACTGTGAATTAGATGCAACATGCAGCATTAA
   60        70        80        90        100       110       120

              100
N  G  R  C  K  Q  F  C  K  R  D  T  D  N  K  V  V  C
GAATGGCAGATGCAAGCAGTTTTGTAAAAGGGACACAGATAACAAGGTGGTTTGT
         130       140       150       160       170

110                        120                        130
S  C  T  D  G  Y  R  L  A  E  D  Q  K  S  C  E  P  A  V  P  F  P
TCCTGTACTGACGGATACCGACTTGCAGAAGACCAAAAGTCCTGCGAACCAGCAGTGCCATTTCC
  180       190       200       210       220       230       240

                         140                        150
C  G  R  V  S  V  S  H [V  R  P  R  F  H  G  L  C  S  C  *  E]
CTGTGGACGAGTCTCTGTCTCACAT[GTGAGGCCCCGCTTTCACGGTCTGTGTTCGTGCTGAGAA] 3'
         250       260      270       280       290       300
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Fig. 5

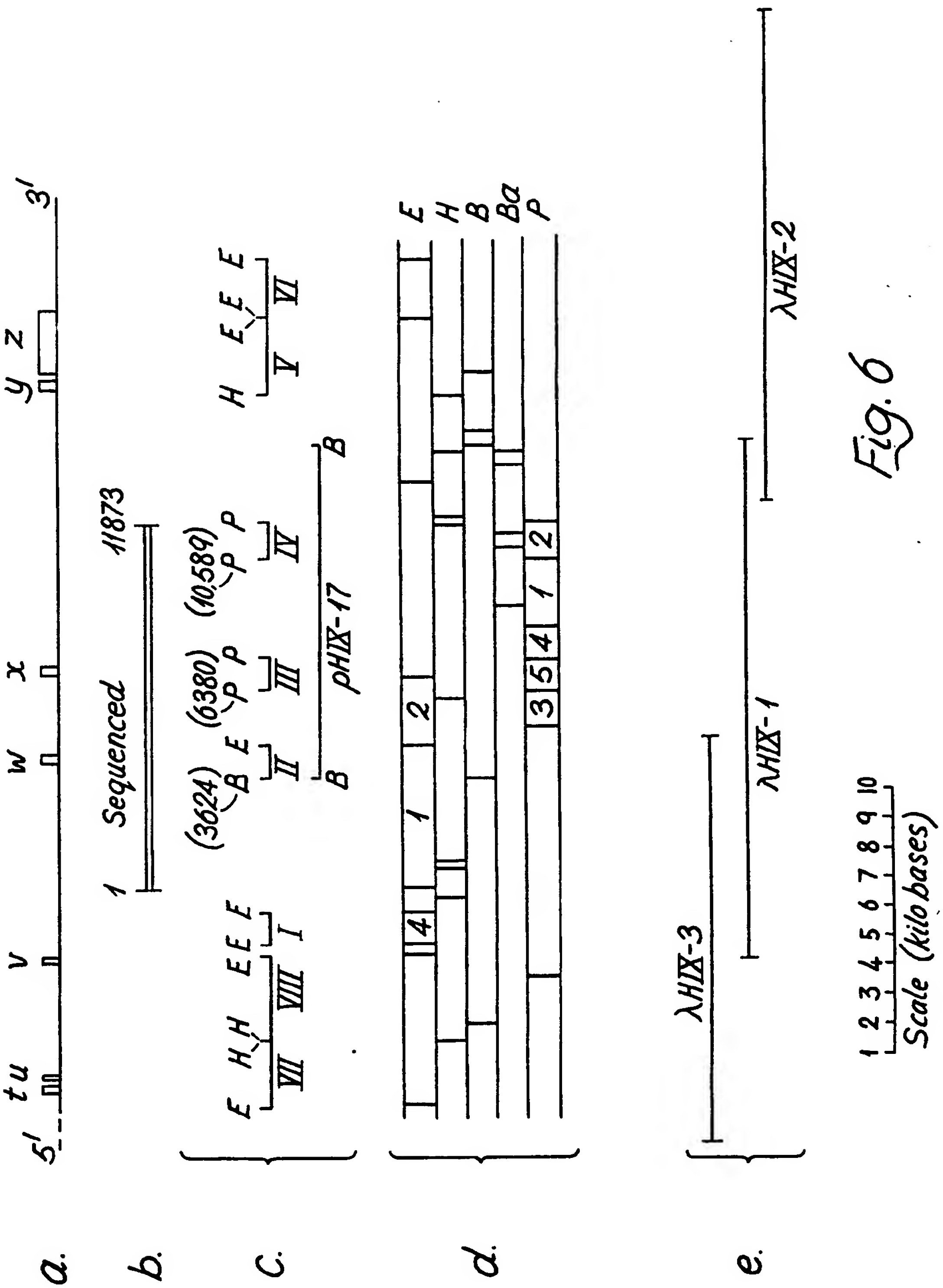
a.
b.
c.
d.
e.
5'
3'
t u
v
w
x
y z
1
Sequenced
11873
(3624)
(6380)
(10,589)
pHIX-17
λHIX-3
λHIX-1
λHIX-2
1 2 3 4 5 6 7 8 9 10
Scale (kilo bases)

Fig. 6

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GAATTCCTTGTGCCATTATTTTATTTCTGGAATCTTCAGCCTTTTAGCTGAGGGCAAAAGATTGCTGATTAGGAAGCAATATTTCCCACCTCCTGCGCAAAACAAGCCAAAGATCAACAG
         10        20        30        40        50        60        70        80        90       100       110       120

CAGCAGCAACATACTGAGCCCTAAAG-GGTGACAAATGTGGAGAATGATACAGAGGTCTGGTTACTTCTTAGCCAATGACACAGAATCACAATTGAGAAAACACAGAGTTTATTCATTCC
        130       140       150       160       170       180       190       200       210       220       230       240

CATTGTGCATGCCCTGACAAACCAAGCTGCACCTTTCGTAACTTATCACAATCTCATATTGACGGAACACTTTCTACAGGTAATGTTTAGTTTGGCTGAACACTTTAGCAATTGCTTCTG
        250       260       270       280       290       300       310       320       330       340       350       360

TAGCAACAAAATGATAGCTAGTAACAGAAAAGTTCAGGAATATTACCACTGTTAGTGAGGAGAAAGGCCTTTTAATTAATTAATTAATTAATTAATAGAACCAAGTACCATCTTTTTG-A
        370       380       390       400       410       420       430       440       450       460       470       480

TCATGCCCTTAGTGAATTATTGGTAGCAAAGGTTAAAGCTCAAGCTGGTTCCTTTGTCCCCTG-CAACAGTTGATTT-CCTCCCTTTATCTCCTGAAGTACCGTAAG-ACTAAGAGCCAA
        490       500       510       520       530       540       550       560       570       580       590       600

TTATTACATTTG-TCATGTCAGCTATATGTAAAATAGAGTTTAAAAGTTTAGATTCATCACTCAAAAATTCATATTCTCCAAAACCATACAGTCACTCTGTTAGCCTGTGTTCCCCCAGA
        610       620       630       640       650       660       670       680       690       700       710       720

AAAAAAGTCACAAGCTTATTTATTAACATGTGCAATCCCA-GGGGCAAGAGAAAGGAACTGAAGAGTGAGGCAGAAAGGAAAAGAAAG-CAATAAGAGGATGAGTTATCAAACTACTCGT
        730       740       750       760       770       780       790       800       810       820       830       840

TTCTAACAGCAACTGATTGCTTAACTTCCTAGGACTGTCTCCAATAAGTCAAATTG-CCTCAGGTTAGCCACCTGAGTGGAAAGAAGCGGTGAAAGAATTTGTCTGTCAGTATCTGTCTC
        850       860       870       880       890       900       910       920       930       940       950       960

TCATTGGTTAGAAGTTCGACTTATGGGGAATTAACTC-CTCACATTTCCTAGTTGGATATGCTTGGGTACAGAGGGT-ATAGTAGCATTACTGCCTCA-GCATGAACAGGGAAGCTTTCA
        970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
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FIG. 7a

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AGGCAAAAGACACATAGTGCAGCTATGAGCCAAGGCAATTCAAGGATACACCCATAGGAGGCTGGTTGACATCCACCCAGAGCTAATCACCACCATGCTGGAAAAAGACACAGGTGAAGC
       1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200

TGAGAAGAATGAAGGTGGTGCATAGGAGGTATCTAATACAGTCACTCATTTTCAAACTTTCCATGTTATGATTGCACTGACCACTGAGGATTTCTATTGAAAGTTTTACTGTTGTCAAAC
       1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320

ACGTACACAAGGGGAAAGGTGTCTTACATTGTTTATGTTCCTGTGCTGCTCTAGAAACAGAAATAGGCTCAAGGCAGAGCCTGTTTTTCTTAATTCAGCAGGTCTAAGCTAACAAGTCCT
       1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440

GAAACATGGTACTTCCTGTTATTGGTATTGCATAGGAGAAACAAAGGGAAAGCACAGTAATTAGAAAATACAAAACAAGATGGCAGGAATAAGCCAAAAATATCAGGAAACACAATTATT
       1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560

GTGAATTGGGATTAAACTAATCTATTAATAATGACAACTTTCAGCTTGGAGTTAAAAATTTAATTGTATACTGTTAACGAAAGTGATACCTAAAATAAAATTACACTGGGAGGCCAAAAT
       1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680

GAAGGGATGTGAAAAGAACTATCAGGTAAAAACTAACAAAAAGAAACTAGCAAAGCAATCTTAATATCAGACAAAATAGATTCAAGAGGAAAATCATTTCAAAAGACAAGAGATTTTTTT
       1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800

TATTAATAAGGGGAATTGCATAGGAGAGTAAAGAAAATGTGGGCCACTGGAATGCTTAGCACTAATGACATATTGGTCTTTGGTCTTCAGTTACCTTACAGGACCCTATTTCATTCTCTT
       1810      1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920

ATGTTTGATATGTAACCACCTCAGCCAGCTTCAAGTTGCTTTTTGGCCCTAATGGACTTCCTAGCACTATAATTTCTTTTTTTTTAAATGTTTTATTTTAGGTTTAGGGGTACATGTGAA
       1930      1940      1950      1960      1970      1980      1990      2000      2010      2020      2030      2040

GGTTTGTTACATAGATAAACATGTGTCACAGGGGTTTGTTGTACATATTATTACATGACGCAGATATTCAGCTCAGTACCAAATAGTGATCTTTTCTGCTCCTCTGCCTCATCCCACCCT
       2050      2060      2070      2080      2090      2100      2110      2120      2130      2140      2150      2160
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FIG. 7b

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CCTCCCTCAAGTAGACTCCAGTATCTGTTGTTTCCTTCTTTGTGTTTATAAGTTCTTAACACTTAGCTCCCGCTTACAAGTGAGAACCTGCAGTATTTGATTTTTGTTCCTACGCTAGTT
      2170      2180      2190      2200      2210      2220      2230      2240      2250      2260      2270      2280

TCCTAAGGATGATAGCCTCCAGCTCCATTCATATTCCCACAAAAGACATAATCTCCTTCTTTTCTATGGCTGCATAATATTCCATGGTATATATGAACCACATTTTCTTTATCCAGTCTG
      2290      2300      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400

TCATTGATGGGCATTTAGGTTGATTCCATGTCTGCTATTCTAACACTGTAATTTCTAAAGACTTCCAGATTCTACTTTTATAGGTAACCTGTTAAACAGTCTAGCTCTGGAAGCCAAGCA
      2410      2420      2430      2440      2450      2460      2470      2480      2490      2500      2510      2520

ATTTCTAGAATAACTAAGCAATAGAAATTACACTTCAATGCAGAAAGGCAGTATCTACATGAGATTATGAAATTGCGGTTGCTTTTTGTGTTCACTGAAAAAAATAAGTAAAACTGTAAC
      2530      2540      2550      2560      2570      2580      2590      2600      2610      2620      2630      2640

TTTCAGAAAAAATGATTGTACATATAGAAAACCCAAAGCATCTAAACAATTAAAATAAATAAGTATAGAAAGATTACTGGATACAGAGTCAACATACAAATATCAATTGTATGTCTATAT
      2650      2660      2670      2680      2690      2700      2710      2720      2730      2740      2750      2760

ACCAGCAACGATTCAAAAATGATTTTTATAATAGCATTAAAAATTAGACGCTTAGTAATAAATGTGAGAAAGATGTGCAAGAACTCTACATAAAAAATTATGAGACGTTATTGAGAAAAA

      2770      2780      2790      2800      2810      2820      2830      2840      2850      2860      2870      2880

TTAAGGAAAACCTAAATAAATGAATAGGCAATGTTTATCAATTAAAGGATACAATATAGTAAATATATCAAATGTTTACTAATGGATTCAATGCAATACCAAAGGTCCCAGCAGGCTTTT
      2890      2900      2910      2920      2930      2940      2950      2960      2970      2980      2990      3000

TTTGGTGGTGGGAGGTCGGGCAGGATTCATAAGCTAATTATAAAATGCATATGGAAATGCAAAGAGCCAAGGATAGCCAAGACAGTTTTGAGGAAGAATAAACTTGTACTACTTACACTA
      3010      3020      3030      3040      3050      3060      3070      3080      3090      3100      3110      3120

CCAGATGTCAAGACTTATTATCGAGTTACATTTATTAAGACAGTGTGGTACTGACACAAGGATAGACAAATAGATCAGTGAAACACACTAGAGTGCTCAGAAGAAGCACACCTGTACATA
      3130      3140      3150      3160      3170      3180      3190      3200      3210      3220      3230      3240

TATAAAGGCTTGATTTATGATAGAGGTGCCAGTGCAGTAGAGAAGGAAATTATTGGTGTTTTCAATAAAAAGTGATAGGTCAATTAGATATTCATATGGCATGAAGTATGAAACAATAAC
      3250      3260      3270      3280      3290      3300      3310      3320      3330      3340      3350      3360
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FIG. 7c

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AATTTATATTCATAACTTGCAGAAAGCAAAAATTTCTTAAAATACAAAAAGTGATCACCATAAAGGAAAAGATTGATAAACTGGACTATATTAAAACTAAGGACTCCTGTTCAGCAAAAG
      3370      3380      3390      3400      3410      3420      3430      3440      3450      3460      3470      3480

ACACTACTTCGACTGAAAAGACAAGTCACAGAGTGAGACAAGATATCTGCAATACAGATACCTAATAACTGAACCCCATACAGTGATGGTGGGAATTTAAGTTCGTACAATCATTTTAGA
      3490      3500      3510      3520      3530      3540      3550      3560      3570      3580      3590      3600

AAATTGCTTGGCAGTAATCTACTAGATCTGAACATGTGATCCAGTAATTACACTCATAATTATAAGCCAGTAAAAAGGCATGTTTATGTCACCAAAAGATATATACAAGAATGTTCATTA
      3610      3620      3630      3640      3650      3660      3670      3680      3690      3700      3710      3720

CACTATTATACATAAGAGCCAAAAACTGGAAACAAACCAAATATCCATTAACAGTAGAATGAATAAATAAAAGCTGTAATAGTAATACAGTGGAATACTACACAGCAATGTAAATGAACT
      3730      3740      3750      3760      3770      3780      3790      3800      3810      3820      3830      3840

ACTGCTGTACAAAACAACATGGTTTAATCTCACAGACAAAATGTTAAATGAAAGACACAGACGAGTACATATTGCGAACTTCTGTTTATAATTCAAGAACTGGCAAGAACTGTTTACTGT
      3850      3860      3870      3880      3890      3900      3910      3920      3930      3940      3950      3960

GTTAGAAGTCCAGGTAATGGTAACCTATAAAAAGGAAAAAGGGTGGAATGATTGGGAGGGGGCATCTTCTGGGGTATTGATAATGTGCTATGTATTGGTCAGTTTAGTGTTTAAACAGGC
      3970      3980      3990      4000      4010      4020      4030      4040      4050      4060      4070      4080

TCATTTACTTTGTGAAAACTTACACTAAAATTGTGTGTATTTTTTGAATATATGTTATACATTAATAAATAGGGTTTTTAAACCTGTAGTTCATAATTTAGTGAAAGTAGAATATCCAAA
      4090      4100      4110      4120      4130      4140      4150      4160      4170      4180      4190      4200

CATTTAGTTTTAAACCAATCAATTATAGTGCTACCATCATTTTTATGCATTATTGAGAAGTTTATTTTACCTTTCTTTCCACTCTTATTTCAAGGCTCCAAAATTTCTCTCCCCAACGTA
      4210      4220      4230      4240      4250      4260      4270      4280      4290      4300      4310      4320

                                                          O
TATTGGGGGCAACATGAATGCCCCCAATGTATATTTGACCCATACATGAGTCAGTAGTTCCATGTACTTTTTAGAAATGCATGTTAAATGATGCTGTTACTGTCTATTTTGCTTCTTTTA
      4330      4340      4350      4360      4370      4380      4390      4400      4410      4420      4430      4440

 J
D   V   T   C   N   I   K   N   G   R   C   E   Q   F   C   K   N   S   A   D   N   K   V   V   C   S   C   T   E   G   Y   R   L   A   E   N   Q   K   S   C
GATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATAACAAGGTGGTTTGCTCCTGTACTGAGGGATATCGACTTGCAGAAAACCAGAAGTCCTGT
      4450      4460      4470      4480      4490      4500      4510      4520      4530      4540      4550      4560
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FIG. 7d

J'

GAACCAGCAGGTCATAATCTGAATAAGATTTTTTAAAGAAAATCTGTATCTGAAACTTCAGCATTTTAACAAACCTACATAATTTTAATTCCTACTTGAATCTGCTTCCTTTTGAAATCA
4570 4580 4590 4600 4610 4620 4630 4640 4650 4660 4670 4680

O'

TAGAAAATATCAGTAGCTTGAATTAGACCAATTAATTTTCTAGATTGCATCATATTTTAAATATAAACTATGTAATCATCTACAACCTGAATTCTTTCTGAGTCCAATTTGTCCAATTTT
4690 4700 4710 4720 4730 4740 4750 4760 4770 4780 4790 4800

TTTCTCTAACATTTATATCACAAAGCAATTAATTTGTGTGATTTCTGCATATGTATTTGTAATTCATCAAGTCAAATCAATGTAGTAATACTATATCATAAAATATACACAAATAATTGA
4810 4820 4830 4840 4850 4860 4870 4880 4890 4900 4910 4920

GTGATAGGCTTCTAGTATAAGGACGGTAAGTTTGAAGCATGATTCTATCTGGGCTGGCTAGTTTACTCTGAGAAAGTTATTTTTTATTGTTGGGTCTTAAGCTGAGTTTACACACTTGGT
4930 4940 4950 4960 4970 4980 4990 5000 5010 5020 5030 5040

GTCAGAATGATTCCGGCAATGAACTGTTTTATGTTCTGCTAGGCTGATCAGCACAATCTATATGGCTGTGAACAAAACAATGTTTCCCAGTCATACCAACCATGCCACCATTTTAACAGC
5050 5060 5070 5080 5090 5100 5110 5120 5130 5140 5150 5160

TGATTAGTGTATTCAGAACATCTCCACTCCATGTTCGTATGGCTGTTATCTAAAGATGAAAGCAGTAGACACTTTTATTTTTTGAAAAATTTAGGCTCTGCAGGGTCAATTATATTTGAT
5170 5180 5190 5200 5210 5220 5230 5240 5250 5260 5270 5280

AAATGAGGGGCTTTTTTGAAGCAAACTAGATATAATTTCTTTTGCATTTCTAAAGCCTGATATCTTATTAATTGGTACATTAAATTGTGCACCATTTCTCTGTAACTGTTTCAGTACCTG
5290 5300 5310 5320 5330 5340 5350 5360 5370 5380 5390 5400

TCTCAGCACTATACCAGGCAGAAGAAATTAAAGAAAAGAACCAGTGCCGAGATCAGCTTGGTCAGGGAGACCCTAATCCTGCGGCACTAGAGGAATTAAAGACACACACACAGAAATATA
5410 5420 5430 5440 5450 5460 5470 5480 5490 5500 5510 5520

GAGTATGAAGTGGGAAATCAGGGGTCTCACAGCCTTCAGAGCTGAGAGCCCCGAACAGAGATTTACCCACATATTTATTGACAGCAAGCCAGTCATAAGATTTACTGAAAGTATTCCTTA
5530 5540 5550 5560 5570 5580 5590 5600 5610 5620 5630 5640

TGGGAAATAAAGGGATGAGTCGGCTAGTTATCTGCAGCAGGAACATGTCCTTAAGGCACAAATCACTTATGCAATTGTCTGTGGTTTAAGAACACCTTTAAGCAGTTTTCCGCCCTGGGT
5650 5660 5670 5680 5690 5700 5710 5720 5730 5740 5750 5760

FIG. 7e

GGGCCAGGTGTTCCTTGCCCTCATTCTGGTAAACCCACAACCTTCCAGTGTGGATATCAAGGCCATCACGAGCATATCACAGTGCTGCAGAGATTTTGTTTATGGCCAGTTTTGGGGCCA
5770 5780 5790 5800 5810 5820 5830 5840 5850 5860 5870 5880

GTTTATGGCCAGATTTGGAGGCCTGTTCCCAACAAACCAGAAGCTAGGAATATATATCCTGCAAATAAAATGAAGAATCTCTAAGGCTTCGGGCCTGCCCACTTGTTCTTCTGCCTGGTT
5890 5900 5910 5920 5930 5940 5950 5960 5970 5980 5990 6000

CTTCACATACACTGTCTCAAAGCTAGTCTACCTTGAGAGGAGCATGAATATGTGTGTGGGTGTGTGTCTGTGTATTTTAACCTTAAAAACCTAACTTCCAGTATAGACAGATGGCATACT
6010 6020 6030 6040 6050 6060 6070 6080 6090 6100 6110 6120

AGCTAAACCCTTACAAGTTCTTCTATGCTATAAAAGAGAAACAGAATTGAGAACCACCTCCAACTATTAAGTGTTATATTTGAATATAGCCTTAGCTTTAGCAGAATAAGTAGGCCAAAC
6130 6140 6150 6160 6170 6180 6190 6200 6210 6220 6230 6240

TTAAAATAAGCTTTTCTGCCTTTTCAATGATAAAGGTCCCTTTTCTGTAGCCATTGTTGATTGTGTACACTTATACATAAGTATTTTGAACTAATTTCCTGTTTTCTCAACCACTTGCTG
6250 6260 6270 6280 6290 6300 6310 6320 6330 6340 6350 6360

TCTTCATGATACTTTGTCGCAGCTGGTTGCTATAGAAATGTCTGTTACAAGGAATGTGGCTTGAAGGAAAGTGATAAATGAAAATGAAATGTGAAGTGACTTTGTTTGACTACAAATTCC

6370 6380 6390 6400 6410 6420 6430 6440 6450 6460 6470 6480

CATTCTGGTAGTCCCCAGTGTATCAATACATTATTTTTCTTTAGAAAATAAACCAACCCAAGGAAAAATGGTGGGCAGGTCCTGGTGAATATGGCTGTGATAATTATATTAGCAATCTCT
6490 6500 6510 6520 6530 6540 6550 6560 6570 6580 6590 6600

TTGGCTAATATTTGAAGCCCAAATAATTGAATCACAATGATCTCTCCCCAGAAAATATATAAAATGCACCTTGGAATCTAGAAGGCCTTTTAGTCTGCAAAAGAAACCTTCTTAATCATA
6610 6620 6630 6640 6650 6660 6670 6680 6690 6700 6710 6720

AGCAGCAGAAGTCCCATTTACCAAATTGGAAAGTTAAAGTTACAAAGCATCAATCATCAGACTTCCATTCAGGGATGGCAATTGGGAGTAAGACTTTTTAGTAAAGAAACTAAACACAAA
6730 6740 6750 6760 6770 6780 6790 6800 6810 6820 6830 6840

FIG. 7f

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GTCATTAGACTCTGTAAAAGTCTTACCAAATTTGATTCTGGAACACCTATTCTATTTCCGTAAAGATGATGAATTCCGGAGCCAAATGTTCTTTTCATGAAGGATTTGAAAACTGTCCAT
    6850      6860      6870      6880      6890      6900      6910      6920      6930      6940      6950      6960

GAAAATAACGCAATCAACCTTTTAGCTTGAGACTCTATTCACTGATTAGATTTTTTAAATACTGATGGGCCTGCTTCTCAGAAGTGACAAGGATGGGCCTCAATCTCAATTTTTGTAAT
    6970      6980      6990      7000      7010      7020      7030      7040      7050      7060      7070      7080

                                                 V  P  F  P  C  G  R  V  S  V  S  Q  T  S  K  L  T  R  A  E  A
ACATGTTCCATTTGCCAATGAGAAATATCAGGTTACTAATTTTTCTTCTATTTTTCTAGTGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGGC
    7090      7100      7110      7120      7130      7140      7150      7160      7170      7180      7190      7200

 V  F  P  D  V  D  Y  V  N  S  T  E  A  E  T  I  L  D  N  I  T  Q  S  T  Q  S  F  N  D  F  T  R  V  V  G  G  E  D  A  K
TGTTTTTCCTGATGTGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACATCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCAA
    7210      7220      7230      7240      7250      7260      7270      7280      7290      7300      7310      7320

 P  G  Q  F  P  W  Q
ACCAGGTCAATTCCCTTGGCAGGTACTTTATACTGATGGTGTGTCAAAACTGGAGCTCAGCTGGCAAGACACAGGCCAGGTGGGAGACTGAGGCTATTTTACTAGACAGACCTATTGGGA
    7330      7340      7350      7360      7370      7380      7390      7400      7410      7420      7430      7440

TGTGAGAAGTATTTAGGCAAGTTTCAGCACTAACCAATGTGAGAAGGCCTCCAGAGATGAGCAGTTGGTGAAAGAGAGGCTCAAAACCAGCTACCATACAGGTCAAGAAGAATTTGGCAT
    7450      7460      7470      7480      7490      7500      7510      7520      7530      7540      7550      7560

TAAGGAAACAGCATAGCAGGATTCCAGACAGGCA---GGTCAACAACATGAAGGTCTGGAAGAAAGGTCGCAGGTACTCAGGTTCAGGGCACTACTTCAGCTTCAGCCCTTGCAAAAACT
    7570      7580      7590      7600      7610      7620      7630      7640      7650      7660      7670      7680

GGTGAGAGTTGGAAAGTCTTTAGGCTAAGAAAAATTGGATTATTTAAAAGGGGGTAAAGAAAGGACTCAAGGAGGAAGGATTAAGGCAAGAACTAGGTT-CAAGAACAGGGCATGAGAG
    7690      7700      7710      7720      7730      7740      7750      7760      7770      7780      7790      7800

AGAGTCTTGATCTACCACTATAGTTCTCGT--------------------------------------------------------------------GGAACTGAACGGAGATTACT
    7810      7820      7830      7840      7850      7860      7870      7880      7890      7900      7910      7920

TAACCGA-ATTTGA-AAC-CCTGG-CAACACG-CGAACCCCACCTCTAATTAAAAAAAATACAAAATTAGCTAGGTGTGATGACTCCCACCTGTGCTCCCAGCTATTCAGGAGGCTGAGG
    7930      7940      7950      7960      7970      7980      7990      8000      8010      8020      8030      8040
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FIG. 7g

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TGGGAGAATCACCTGAGCCTGGAAAGTCGAGGCTGCAGTGAATTGTGATCACACCACTGCACTTCAGCCTGAGTGACAGAGTAAGACCCTATCTCAAAAAACAGAAAAAGAAAAACACTG
      8050      8060      8070      8080      8090      8100      8110      8120      8130      8140      8150      8160

GCCCAAAGGAAATGAACTTGTTACAGAAGCCGGGGTTCAAAACACCAAATAATGCACTTGTACCTAGTCCTTCCCGGGTGCTCTGCAGACATTTCTCCAAGCGTAGTCTGCAAACAACCT
      8170      8180      8190      8200      8210      8220      8230      8240      8250      8260      8270      8280

ACATATGTAGAATTACCTATGCACATTTTTCATTTAACAACCAAG-GCTACATTTGTAGCAAAATCTGGGTTGTAACTTAGCCTACAGCTGAAGCCTAAGAGATTCCGTCTGTGAGAAGA
      8290      8300      8310      8320      8330      8340      8350      8360      8370      8380      8390      8400

AATAACCCACCTCTTTGGCCCCCCTCCCCAGGCAGGAAGCCAGGATGGTCCTTATATAAAGTTGTGCTGT-CAATAGGTAACCACTAGCCACATATG--TTTAAATTTAAATTAACTACA
      8410      8420      8430      8440      8450      8460      8470      8480      8490      8500      8510      8520

ATTAAGAGAAATTAAAAATTCAATTC-TCAATTGCACCTGCCAAATTTTAAGCACATAACAACCACATGTGG-TAGTAACTACTGTATTGGAGAGTGCAAGCGGAGATAGAACACTCTAT
      8530      8540      8550      8560      8570      8580      8590      8600      8610      8620      8630      8640

TACTGCAGAAATTTCTATTGGATAGCAGTTATAATAGTTTAGTGTAACTTAAAACT-CCTAGTTGCCACAAGTCATGATTTAGTAGTAATTTCATGGA----------------------
      8650      8660      8670      8680      8690      8700      8710      8720      8730      8740      8750      8760

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      8770      8780      8790      8800      8810      8820      8830      8840      8850      8860      8870      8880

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      8890      8900      8910      8920      8930      8940      8950      8960      8970      8980      8990      9000

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      9010      9020      9030      9040      9050      9060      9070      9080      9090      9100      9110      9120

----------------------------------------------------------------------------------AAAAGACAATATTTGCTG-ACCGATCTTATAACTCATAAATG
      9130      9140      9150      9160      9170      9180      9190      9200      9210      9220      9230      9240
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FIG. 7h

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G-ACACTGTATGTTCCTTTTTACCTCCTCTGTTTCTACTTAATTGCACCCTATGAGGACTGCTTCCCTTACCTACCATAACCCCTTCCTTCACTCATCCATATCTTTACTCTTCTTCACA
         9250      9260      9270      9280      9290      9300      9310      9320      9330      9340      9350      9360

ACTCTGTAATATTGACCTTCTTTA-GAACCTTTCCTGGAACAATCCCTCTTAAGTGCAAGCACTGTTATTATGCCTTCAATGTATTTAATATCCATGTATCTATTCTCTCTAATTTTGTC
         9370      9380      9390      9400      9410      9420      9430      9440      9450      9460      9470      9480

ATTTTGTGTTCTCATGTATTTTCATTCATTATGTGTCCAACTTCCATGGATAACATGGTTACAACAAAAGATCCTACTTTATGACAATTATCTTCCTTGGGTTTGTGGGACATAGAACAG
         9490      9500      9510      9520      9530      9540      9550      9560      9570      9580      9590      9600

TGCTCAGAGTAGGGGATCCAAGAACCCAGGAGAATATATTAGCTAAGAAGATAACTTCCGTTTTTAAAAGTCCAAGATTCAGGAGATCAAAACCATCCTGGCTAACATAGTGAAACCCCG
         9610      9620      9630      9640      9650      9660      9670      9680      9690      9700      9710      9720

TCTCTTCCAAAAATACAAAAAATTAGCCCGGCGTGGTGGCAGGCGCCTATAGTCCCAGCTACACGGGAGGCTGAGGCAGGAGAATGGCGTGAACCGGGGAGGCGGAGCTGGCAGTGAGCC
         9730      9740      9750      9760      9770      9780      9790      9800      9810      9820      9830      9840

GAGATCCCGCCACTGCACTCCAGCCTGGGCGACAGAGCGAGACTCC---AAAAAAAAAAAAAAAAAAAAGTCCAAGTTTAAAAAAAAAAAAAAAAAAAGGTGT-TGT-TTGTGAGTTT--
         9850      9860      9870      9880      9890      9900      9910      9920      9930      9940      9950      9960

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         9970      9980      9990     10000     10010     10020     10030     10040     10050     10060     10070     10080

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        10090     10100     10110     10120     10130     10140     10150     10160     10170     10180     10190     10200

--------CCCTATTCAACCACATGAACAGATTACTGATGTGACAGATTCAAAGCACTTTATCTTTCCAAAGGCAAGAAGCTGAGCTACTTTCCAGAATAGTTGTGAAAGACCCTGTCAT
        10210     10220     10230     10240     10250     10260     10270     10280     10290     10300     10310     10320
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FIG. 7i

ACTTCTGCATTGTTTCCTCCACACCACCTCCATCCAGTTCCTTATGAATGGTTACTGGTTTTCAAAAATATGAGATAAATTGAGTGTATAAAAGTCATTTTTAGACAAAATGAAACAGGA
10330 10340 10350 10360 10370 10380 10390 10400 10410 10420 10430 10440

AATGAAAGAAACCAGAATCTCTCCTCATTTGTGGATGGGCCAGCTCCACCATGTCATGGTTAATCTGCAGGGAGGAAATACTAGATTTGATTGCAGATCAGACTGCAGCAAACCTGCTGT
10450 10460 10470 10480 10490 10500 10510 10520 10530 10540 10550 10560

GACTAAGGCATCAAGAGAAAGCAAGCAACAGCTGGGGCTTCAGTGGTGAAAACATTATATATCTAGCTTTGAATATGAAATACTGTTTAGCAGTGTCACCTAGAAAAGAGTGTTTCAAAA
10570 10580 10590 10600 10610 10620 10630 10640 10650 10660 10670 10680

TGCTGATGCAACCTTTCTCTTCAGAGTTGTTTCTTTTATCTTTCAAATTTAGCCAGGGTGGGAAATAAAGTGATCACTTGGTGAAGAAATCTCACAAAGAAGAACATAGAGAGTTCACTT
10690 10700 10710 10720 10730 10740 10750 10760 10770 10780 10790 10800

TCATCTGGAGTAATGAACAGATTGAACAAACTAGAAATGGTTAGTCTGTTAAAGAAAAGGTGTAGGTGAGCTGTTTGCAAGAGCCACAAGGGAAAGGGGAAGACAACTTCTTTGTGGACT
10810 10820 10830 10840 10850 10860 10870 10880 10890 10900 10910 10920

TAAGGGTGAAAGTTGCAAGCAGGCAAGACGATTCTGACCTCCATTAAGAAAGCCCTTTCCAACCAACAACCACTGGGTTGGTTACACAGGTTGGGCAGCATTGGGAGCAAATGTTGATTG
10930 10940 10950 10960 10970 10980 10990 11000 11010 11020 11030 11040

AACAAATGTTTGTCGGAATTGTTGACTTAAAGAGCTGTTCTGTCACTGGGGACAGCGGCTAGATAGCCCCATTCAGGGAG-GGGCATTTGTTCACCTGGCCAGAGATCAGAGCAGGCTAA
11050 11060 11070 11080 11090 11100 11110 11120 11130 11140 11150 11160

GG-ACT-CTGGATCCTGTCCAGCTTTGAGACCCTACAGAGCCATGTTCTCCTAGCACGTATCCCGTCTGCGGTCACGGTCATTTCTTACCTTATTCCAGGGCTTTCACCTCAGCTTGCCA
11170 11180 11190 11200 11210 11220 11230 11240 11250 11260 11270 11280

GGCTGGAGCCAAGGGCAACGCAGCCGC-CTTGTTCGCGATGGTAGCTTCCCAGGAGCCCCCTATGGTTCCGGAACGGCGCTG--CCCATCCTGTTTGCTACCTCCTAAAGCCAAAGG--C
11290 11300 11310 11320 11330 11340 11350 11360 11370 11380 11390 11400

TGGCGGG-C-GG-C---CTTCTAAAGTCGCGCAAGGTTAGAAGGTTCCGGACAGGAACGGCGTGAGGCCAATGGAAGGAGGTACTTCAGTTTCCCTCCAGGCCCGCGCGATGGGCTCAGA
11410 11420 11430 11440 11450 11460 11470 11480 11490 11500 11510 11520

FIG. 7j

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GCTCCTTGAGAACTCGGGAAAGGAAGCAGGGTCTCTGAAGAAATACTTCAGGAGTAGAAAGAGGAAGCTAGAGGGTTAAATGCACTACACAGGAACAGAAATGAGTTTTTCTTAGAGTTA
      11530     11540     11550     11560     11570     11580     11590     11600     11610     11620     11630     11640

GTATATGTCTAGAGGTGTAGTAAACTAAAACAAGTCTTGAATTGCATACCGCCACGTAGGGAAGAAATGAAAACCTTTGAATATTAGTGAAAAAAGGGAAACTGCAACGCCTGTATTACT
      11650     11660     11670     11680     11690     11700     11710     11720     11730     11740     11750     11760

AGATAGCTTTCATCAACAGCTCAAAACCGACAGATTTAAAGAAGCAACACCGCATTTTGGCTTTCTAAAGCTTTAATTTGGTTTGGATCCCATGCCCATGACCCTGCCAGCTG

      11770     11780     11790     11800     11810     11820     11830     11840     11850     11860     11870
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FIG. 7k

FIG. 8(a)

→ 1	0.000	ECOR1	GAATTC
30	0.002	HINF1	GAATC
33	0.003	MBO11	TCTTC
46	0.004	ALU1	AGCT
48	0.004	DDE1	CTGAG
50	0.004	MNL1	GAGG
89	0.007	MNL1	CCTC
94	0.008	MST1	TGCGCA
95	0.008	HHA1	GCGC
112	0.009	MBO1	GATC
120	0.010	BBV1	GCAGC
120	0.010	FNU4H1	GCAGC
123	0.010	BBV1	GCAGC
123	0.010	FNU4H1	GCAGC
134	0.011	DDE1	CTGAG
148	0.012	HPH1	GGTGA
173	0.014	MNL1	GAGG
188	0.016	DDE1	CTTAG
204	0.017	HINF1	GAATC
247	0.021	SPH1	GCATGC
265	0.022	ALU1	AGCT
266	0.022	BBV1	GCTGC
266	0.022	FNU4H1	GCTGC
305	0.026	XMN1	GAACACTTTC
376	0.032	ALU1	AGCT
417	0.035	MNL1	GAGG
425	0.036	STU1	AGGCCT
426	0.036	HAE111	GGCC
465	0.039	RSA1	GTAC
488	0.041	DDE1	CTTAG
517	0.043	ALU1	AGCT
523	0.044	ALU1	AGCT
559	0.047	MNL1	CCTC
578	0.049	RSA1	GTAC
590	0.050	DDE1	CTAAG
621	0.052	ALU1	AGCT
652	0.055	HINF1	GATTC
→ 732	0.062	HIND111	AAGCTT
733	0.062	ALU1	AGCT
781	0.066	MBO11	GAAGA
788	0.066	MNL1	GAGG
816	0.069	MNL1	GAGG

FIG. 8(b)

818	0.069	FOK1	GGATG
898	0.076	MNL1	CCTC
898	0.076	MST11	CCTCAGG
899	0.076	DDE1	CTCAG
913	0.077	DDE1	CTGAG
929	0.078	HPH1	GGTGA
976	0.082	TAQ1	TCGA
1027	0.086	RSA1	GTAC
1032	0.087	MNL1	GAGG
1054	0.089	MNL1	CCTC
1072	0.090	HIND111	AAGCTT
1073	0.090	ALU1	AGCT
1099	0.092	BBV1	GCAGC
1099	0.092	FNU4H1	GCAGC
1101	0.093	ALU1	AGCT
1138	0.096	MNL1	GAGG
1145	0.096	HINC11	GTTGAC
1150	0.097	FOK1	CATCC
1161	0.098	ALU1	AGCT
1167	0.098	HPH1	TCACC
1193	0.100	HPH1	GGTGA
1198	0.101	ALU1	AGCT
1200	0.101	DDE1	CTGAG
1204	0.101	MBO11	GAAGA
1226	0.103	MNL1	GAGG
1284	0.108	DDE1	CTGAG
1286	0.108	MNL1	GAGG
1323	0.111	RSA1	GTAC
1365	0.115	BBV1	GCTGC
1365	0.115	FNU4H1	GCTGC
1370	0.115	XBA1	TCTAGA
1424	0.120	DDE1	CTAAG
1427	0.120	ALU1	AGCT
1449	0.122	RSA1	GTAC
1603	0.135	ALU1	AGCT
1626	0.137	ACC1	GTATAC
1633	0.137	HINC11	GTTAAC
1633	0.137	HPA1	GTTAAC
1670	0.141	MNL1	GAGG
1672	0.141	HAE111	GGCC
1685	0.142	FOK1	GGATG
1759	0.148	HINF1	GATTC
1766	0.149	MNL1	GAGG
1841	0.155	SAU961	GGGCC
1842	0.155	HAE111	GGCC

FIG. 8(c)

1855	0.156	DDE1	CTTAG
1884	0.159	MBO11	TCTTC
1901	0.160	AVA11	GGACC
1901	0.160	SAU961	GGACC
1939	0.163	MNL1	CCTC
1940	0.163	DDE1	CTCAG

1947	0.164	ALU1	AGCT
1965	0.165	HAE111	GGCC
1965	0.165	SAU961	GGCCC
2030	0.171	RSA1	GTAC
2081	0.175	RSA1	GTAC
2097	0.177	HGA1	GACGC
2110	0.178	ALU1	AGCT
2112	0.178	DDE1	CTCAG
2116	0.178	RSA1	GTAC
2128	0.179	MBO1	GATC
2141	0.180	MNL1	CCTC
2147	0.181	MNL1	CCTC
2150	0.181	FOK1	CATCC
2158	0.182	MNL1	CCTC
2161	0.182	MNL1	CCTC
2165	0.182	MNL1	CCTC
2171	0.183	ACC1	GTAGAC
2174	0.183	HINF1	GACTC
2222	0.187	DDE1	CTTAG
2225	0.187	ALU1	AGCT
2248	0.189	PST1	CTGCAG
2282	0.192	MST11	CCTAAGG
2283	0.192	DDE1	CTAAG
2287	0.193	FOK1	GGATG
2296	0.193	MNL1	CCTC
2301	0.194	ALU1	AGCT
2349	0.198	BBV1	GCTGC
2349	0.198	FNU4H1	GCTGC
2422	0.204	HINF1	GATTC
2468	0.208	HINF1	GATTC
2483	0.209	BSTE11	GGTAACC
2503	0.211	ALU1	AGCT
2524	0.212	XBA1	TCTAGA
2534	0.213	DDE1	CTAAG

FIG. 8(d)

2658	0.224	RSA1	GTAC
2678	0.225	SFNA1	GCATC
2726	0.230	HINF1	GAGTC
2728	0.230	HINC11	GTCAAC
2770	0.233	HINF1	GATTC
2807	0.236	HGA1	GACGC
2811	0.237	DDE1	CTTAG
2965	0.250	HINF1	GATTC
2984	0.251	AVA11	GGTCC
2984	0.251	SAU961	GGTCC
3012	0.254	MNL1	GAGG
3024	0.255	HINF1	GATTC
3032	0.255	ALU1	AGCT
3048	0.257	NDE1	CATATG
3090	0.260	MNL1	GAGG
3093	0.260	MBO11	GAAGA
3106	0.262	RSA1	GTAC
3141	0.264	TAQ1	TCGA
3168	0.267	RSA1	GTAC
3193	0.269	MBO1	GATC
3213	0.271	HGIA1	GTGCTC
3216	0.271	DDE1	CTCAG
3220	0.271	MBO11	GAAGA
3234	0.272	RSA1	GTAC
3263	0.275	MNL1	GAGG
3333	0.281	NDE1	CATATG
3412	0.287	BCL1	TGATCA
3413	0.287	MBO1	GATC
3415	0.288	HPH1	TCACC
3457	0.291	DDE1	CTAAG
3462	0.292	HINF1	GACTC
3489	0.294	TAQ1	TCGA
3522	0.297	ECOR5	GATATC
3585	0.302	RSA1	GTAC
→ 3624	0.305	BGL11	AGATCT
3625	0.305	MBO1	GATC
3638	0.306	MBO1	GATC
3689	0.311	HPH1	TCACC
3792	0.319	ALU1	AGCT

FIG. 8(e)

3847	0.324	RSA1	GTAC
3905	0.329	RSA1	GTAC
3970	0.334	BSTN1	CCAGG
3970	0.334	SCRF1	CCAGG
3979	0.335	BSTE11	GGTAACC
4016	0.338	MNL1	GAGG
4022	0.339	SFNA1	GCATC
4025	0.339	MBO11	TCTTC
4368	0.368	HINF1	GAGTC
4384	0.369	RSA1	GTAC
4410	0.371	SFNA1	GATGC
4469	0.376	SFNA1	GATGC
4520	0.381	RSA1	GTAC
4523	0.381	DDE1	CTGAG
4525	0.381	MNL1	GAGG
4529	0.381	ECOR5	GATATC
4533	0.382	TAQ1	TCGA
4658	0.392	HINF1	GAATC
4695	0.395	ALU1	AGCT
4719	0.397	XBA1	TCTAGA
4727	0.398	SFNA1	GCATC
4769	0.402	ECOR1	GAATTC
4769	0.402	XMN1	GAATTCTTTC
4778	0.402	DDE1	CTGAG
4780	0.403	HINF1	GAGTC
4848	0.408	NDE1	CATATG
4961	0.418	HINF1	GATTC
4988	0.420	DDE1	CTGAG
5020	0.423	ALU1	AGCT
5022	0.423	DDE1	CTGAG
5049	0.425	HINF1	GATTC
5053	0.426	HPA11	CCGG
5085	0.428	BCL1	TGATCA
5086	0.428	MBO1	GATC
5157	0.434	PVU11	CAGCTG
5158	0.434	ALU1	AGCT
5225	0.440	ACC1	GTAGAC
5258	0.443	PST1	CTGCAG
5285	0.445	MNL1	GAGG
5339	0.450	ECOR5	GATATC
5355	0.451	RSA1	GTAC
5367	0.452	HGIA1	GTGCAC
5394	0.454	RSA1	GTAC
5402	0.455	DDE1	CTCAG
5414	0.456	BSTN1	CCAGG

FIG. 8(f)

5414	0.456	SCRF1	CCAGG
5421	0.456	MBO11	GAAGA
5451	0.459	MBO1	GATC
5455	0.459	ALU1	AGCT

5481	0.462	FNU4H1	GCGGC
5490	0.462	MNL1	GAGG
5560	0.468	ALU1	AGCT
5562	0.468	DDE1	CTGAG
5627	0.474	XMN1	GAAAGTATTC
5653	0.476	FOK1	GGATG
5657	0.476	HINF1	GAGTC
5672	0.478	PST1	CTGCAG
5674	0.478	BBV1	GCAGC
5674	0.478	FNU4H1	GCAGC
5754	0.485	BSTN1	CCTGG
5754	0.485	SCRF1	CCTGG
5761	0.485	SAU961	GGGCC
5762	0.485	HAE111	GGCC
5764	0.485	BSTN1	CCAGG
5764	0.485	SCRF1	CCAGG
5779	0.487	MNL1	CCTC
5813	0.490	ECOR5	GATATC
5821	0.490	HAE111	GGCC
5844	0.492	BBV1	GCTGC
5844	0.492	FNU4H1	GCTGC
5845	0.492	PST1	CTGCAG
5863	0.494	BAL1	TGGCCA
5864	0.494	HAE111	GGCC
5875	0.495	SAU961	GGGCC
5876	0.495	HAE111	GGCC
5886	0.496	BAL1	TGGCCA
5887	0.496	HAE111	GGCC
5898	0.497	MNL1	GAGG
5899	0.497	STU1	AGGCCT
5900	0.497	HAE111	GGCC
5922	0.499	ALU1	AGCT
5952	0.501	MBO11	GAAGA
5955	0.501	HINF1	GAATC
5961	0.502	DDE1	CTAAG
5971	0.503	SAU961	GGGCC

FIG. 8(g)

5972	0.503	HAE111	GGCC
5987	0.504	MBO11	TCTTC
5994	0.505	BSTN1	CCTGG
5994	0.505	SCRF1	CCTGG
6000	0.505	MBO11	TCTTC
6021	0.507	ALU1	AGCT
6026	0.507	ACC1	GTCTAC
6037	0.508	MNL1	GAGG
6121	0.515	ALU1	AGCT
6139	0.517	MBO11	TCTTC
6177	0.520	MNL1	CCTC
6211	0.523	DDE1	CTTAG
6214	0.523	ALU1	AGCT
6233	0.525	HAE111	GGCC
→ 6248	0.526	HIND111	AAGCTT
6249	0.526	ALU1	AGCT
6275	0.528	AVA11	GGTCC
6275	0.528	SAU961	GGTCC
6305	0.531	RSA1	GTAC
6361	0.536	MBO11	TCTTC
6379	0.537	BBV1	GCAGC
6379	0.537	FNU4H1	GCAGC
→ 6380	0.537	PVU11	CAGCTG
6381	0.537	ALU1	AGCT
6558	0.552	AVA11	GGTCC
6558	0.552	SAU961	GGTCC
6561	0.553	BSTN1	CCTGG
6561	0.553	SCRF1	CCTGG
6564	0.553	HPH1	GGTGA
6629	0.558	HINF1	GAATC
6639	0.559	MBO1	GATC
6674	0.562	HINF1	GAATC
6677	0.562	XBA1	TCTAGA
6683	0.563	STU1	AGGCCT
6684	0.563	HAE111	GGCC
6722	0.566	BBV1	GCAGC
6722	0.566	FNU4H1	GCAGC
6767	0.570	SFNA1	GCATC
6793	0.572	FOK1	GGATG
6848	0.577	HINF1	GACTC

FIG. 8(h)

6874	0.579	HINF1	GATTC
6911	0.582	ECOR1	GAATTC
6916	0.582	HPA11	CCGG
6984	0.588	ALU1	AGCT
6991	0.589	HINF1	GACTC
7028	0.592	SAU961	GGGCC
7029	0.592	HAE111	GGCC
7038	0.593	DDE1	CTCAG
7052	0.594	FOK1	GGATG
7056	0.594	SAU961	GGGCC
7057	0.594	HAE111	GGCC
7059	0.594	MNL1	CCTC
7124	0.600	MBO11	TCTTC
7155	0.603	MBO11	GAAGA
7155	0.603	XMN1	GAAGAGTTTC
7179	0.605	DDE1	CTAAG
7182	0.605	ALU1	AGCT
7185	0.605	HPH1	TCACC
7194	0.606	DDE1	CTGAG
7196	0.606	MNL1	GAGG
7237	0.609	ALU1	AGCT
7293	0.614	AVA1	CTCGGG
7310	0.616	MBO11	GAAGA
7313	0.616	SFNA1	GATGC
7322	0.617	BSTN1	CCAGG
7322	0.617	SCRF1	CCAGG
7343	0.618	RSA1	GTAC
7373	0.621	HGIA1	GAGCTC
7373	0.621	SAC1	GAGCTC
7374	0.621	ALU1	AGCT
7376	0.621	DDE1	CTCAG
→ 7378	0.621	PVU11	CAGCTG
7379	0.621	ALU1	AGCT
7394	0.623	HAE111	GGCC
7396	0.623	BSTN1	CCAGG
7396	0.623	SCRF1	CCAGG
7408	0.624	DDE1	CTGAG
7410	0.624	MNL1	GAGG
7438	0.626	FOK1	GGATG
7485	0.630	STU1	AGGCCT
7486	0.630	HAE111	GGCC
7488	0.631	MNL1	CCTC
7507	0.632	HPH1	GGTGA
7516	0.633	MNL1	GAGG
7529	0.634	ALU1	AGCT
7547	0.636	MBO11	GAAGA

FIG. 8(i)

7580	0.638	HINF1	GATTC
7599	0.640	HINC11	GTCAAC
7619	0.642	MBO11	GAAGA
7634	0.643	RSA1	GTAC
7637	0.643	DDE1	CTCAG
7659	0.645	ALU1	AGCT
7681	0.647	HPH1	GGTGA
7705	0.649	DDE1	CTAAG
7745	0.652	HINF1	GACTC
7753	0.653	MNL1	GAGG
7802	0.657	HINF1	GAGTC
7809	0.658	MBO1	GATC
7940	0.669	BSTN1	CCTGG
7940	0.669	SCRF1	CCTGG
7963	0.671	MNL1	CCTC
7989	0.673	ALU1	AGCT
8002	0.674	HINF1	GACTC
8013	0.675	HGIA1	GTGCTC
8021	0.675	ALU1	AGCT
8031	0.676	MNL1	GAGG
8035	0.677	DDE1	CTGAG
8037	0.677	MNL1	GAGG
8046	0.678	HINF1	GAATC
8049	0.678	HPH1	TCACC
8053	0.678	DDE1	CTGAG
8058	0.679	BSTN1	CCTGG
8058	0.679	SCRF1	CCTGG
8067	0.679	TAQ1	TCGA
8069	0.680	MNL1	GAGG
8072	0.680	BBV1	GCTGC
8072	0.680	FNU4H1	GCTGC
8073	0.680	PST1	CTGCAG
8086	0.681	BCL1	TGATCA
8087	0.681	MBO1	GATC
8109	0.683	DDE1	CTGAG
8160	0.687	HAE111	GGCC
8160	0.687	SAU961	GGCCC
8190	0.690	HPA11	CCGG

FIG. 8(j)

8190	0.690	NCI1	CCGGG
8190	0.690	SCRF1	CCGGG
8220	0.692	RSA1	GTAC
8233	0.693	AVA1	CCCGGG
8233	0.693	NCI1	CCCGG
8233	0.693	SCRF1	CCCGG
8233	0.693	SMA1	CCCGGG
8234	0.693	HPA11	CCGG
8234	0.693	NCI1	CCGGG
8234	0.693	SCRF1	CCGGG
8238	0.694	HGIA1	GTGCTC
8243	0.694	PST1	CTGCAG
8282	0.697	NDE1	CATATG
8357	0.704	DDE1	CTTAG
8366	0.705	PVU11	CAGCTG
8367	0.705	ALU1	AGCT
8376	0.705	DDE1	CTAAG
8382	0.706	HINF1	GATTC
8396	0.707	MBO11	GAAGA
8410	0.708	MNL1	CCTC
8417	0.709	HAE111	GGCC
8417	0.709	SAU961	GGCCC
8423	0.709	MNL1	CCTC

8428	0.710	BSTN1	CCAGG
8428	0.710	SCRF1	CCAGG
8440	0.711	BSTN1	CCAGG
8440	0.711	SCRF1	CCAGG
8443	0.711	FOK1	GGATG
8447	0.711	AVA11	GGTCC
8447	0.711	SAU961	GGTCC
8477	0.714	BSTE11	GGTAACC
8492	0.715	NDE1	CATATG
8643	0.728	PST1	CTGCAG
9221	0.777	MBO1	GATC
9263	0.780	MNL1	CCTC
9266	0.780	MNL1	CCTC
9294	0.783	MNL1	GAGG
9335	0.786	FOK1	CATCC
9350	0.787	MBO11	TCTTC

FIG. 8(k)

9353	0.788	MBO11	TCTTC
9394	0.791	BSTN1	CCTGG
9394	0.791	SCRF1	CCTGG
9406	0.792	MNL1	CCTC
9550	0.804	MBO1	GATC
9571	0.806	MBO11	TCTTC
9600	0.808	HGIA1	GTGCTC
9603	0.809	DDE1	CTCAG
→ 9614	0.810	BAMH1	GGATCC
9615	0.810	MBO1	GATC
9626	0.811	BSTN1	CCAGG
9626	0.811	SCRF1	CCAGG
9641	0.812	ALU1	AGCT
9643	0.812	DDE1	CTAAG
9647	0.812	MBO11	GAAGA
9676	0.815	HINF1	GATTC
9685	0.816	MBO1	GATC
9694	0.816	FOK1	CATCC
9697	0.817	BSTN1	CCTGG
9697	0.817	SCRF1	CCTGG
9723	0.819	MBO11	TCTTC
9747	0.821	NCI1	CCCGG
9747	0.821	SCRF1	CCCGG
9748	0.821	HPA11	CCGG
9762	0.822	HAE11	GGCGCC
9762	0.822	NAR1	GGCGCC
9763	0.822	HHA1	GCGC
9777	0.823	ALU1	AGCT
9787	0.824	MNL1	GAGG
9791	0.825	DDE1	CTGAG
9793	0.825	MNL1	GAGG
9814	0.826	HPA11	CCGG
9814	0.826	NCI1	CCGGG
9814	0.826	SCRF1	CCGGG
9819	0.827	MNL1	GAGG
9826	0.828	ALU1	AGCT
9843	0.829	MBO1	GATC
9864	0.831	BSTN1	CCTGG
9864	0.831	SCRF1	CCTGG
9881	0.832	HINF1	GACTC
10246	0.863	HINF1	GATTC
10279	0.866	ALU1	AGCT
10281	0.866	DDE1	CTGAG
10284	0.866	ALU1	AGCT
10310	0.868	TTH1111	GACCCTGTC

FIG. 8(L)

10336	0.870	MNL1	CCTC
10347	0.871	MNL1	CCTC
10351	0.872	FOK1	CATCC
10455	0.880	HINF1	GAATC
10463	0.881	MNL1	CCTC
10473	0.882	FOK1	GGATG
10477	0.882	SAU961	GGGCC
10478	0.882	HAE111	GGCC
10482	0.883	ALU1	AGCT
10505	0.885	PST1	CTGCAG
10512	0.885	MNL1	GAGG
10536	0.887	MBO1	GATC
10543	0.888	PST1	CTGCAG
10545	0.888	BBV1	GCAGC
10545	0.888	FNU4H1	GCAGC
10563	0.890	DDE1	CTAAG
10568	0.890	SFNA1	GCATC
10589	0.892	PVU11	CAGCTG
10590	0.892	ALU1	AGCT
10605	0.893	HPH1	GGTGA
10625	0.895	ALU1	AGCT
10656	0.897	HPH1	TCACC
10685	0.900	SFNA1	GATGC
10698	0.901	MBO11	TCTTC
10733	0.904	BSTN1	CCAGG
10733	0.904	SCRF1	CCAGG
10751	0.905	BCL1	TGATCA
10752	0.905	MBO1	GATC
10760	0.906	HPH1	GGTGA
10763	0.906	MBO11	GAAGA
10779	0.908	MBO11	GAAGA
10865	0.915	HPH1	GGTGA
10869	0.915	ALU1	AGCT
10899	0.918	MBO11	GAAGA
10925	0.920	HPH1	GGTGA
10950	0.922	HINF1	GATTC
10958	0.923	MNL1	CCTC
11015	0.928	BBV1	GCAGC

FIG. 8(m)

11015	0.928	FNU4H1	GCAGC
11061	0.932	HINC11	GTTGAC
11073	0.933	ALU1	AGCT
11095	0.934	FNU4H1	GCGGC
11132	0.938	HPH1	TCACC
11135	0.938	BSTN1	CCTGG
11135	0.938	SCRF1	CCTGG
11137	0.938	BAL1	TGGCCA
11138	0.938	HAE111	GGCC
11145	0.939	MBO1	GATC
11157	0.940	DDE1	CTAAG
11170	0.941	BAMH1	GGATCC
11171	0.941	MBO1	GATC
11181	0.942	ALU1	AGCT
11256	0.948	BSTN1	CCAGG
11256	0.948	SCRF1	CCAGG
11265	0.949	HPH1	TCACC
11268	0.949	MNL1	CCTC
11269	0.949	DDE1	CTCAG
11272	0.949	ALU1	AGCT
11278	0.950	BSTN1	CCAGG
11278	0.950	SCRF1	CCAGG
11300	0.952	BBV1	GCAGC

11300	0.952	FNU4H1	GCAGC
11303	0.952	FNU4H1	GCCGC
11314	0.953	NRU1	TCGCGA
11315	0.953	FNUD11	CGCG
11324	0.954	ALU1	AGCT
11330	0.954	BSTN1	CCAGG
11330	0.954	SCRF1	CCAGG
11349	0.956	HPA11	CCGG
11356	0.956	HAE11	GGCGCT
11357	0.956	HHA1	GCGC
11367	0.957	FOK1	CATCC
11381	0.958	MNL1	CCTC
11428	0.962	FNUD11	CGCG
11429	0.963	HHA1	GCGC
11447	0.964	HPA11	CCGG
11464	0.965	MNL1	GAGG

FIG. 8(n)

11466	0.966	HAE111	GGCC
11478	0.967	MNL1	GAGG
11481	0.967	RSA1	GTAC
11494	0.968	MNL1	CCTC
11497	0.968	BSTN1	CCAGG
11497	0.968	SCRF1	CCAGG
1150C	0.968	HAE111	GGCC
11500	0.968	SAU961	GGCCC
11504	0.969	FNUD11	CGCG
11505	0.969	HHA1	GCGC
11506	0.969	FNUD11	CGCG
11515	0.970	DDE1	CTCAG
11519	0.970	HGIA1	GAGCTC
11519	0.970	SAC1	GAGCTC
11520	0.970	ALU1	AGCT
11533	0.971	AVA1	CTCGGG
11557	0.973	MBO11	GAAGA
11560	0.974	XMN1	GAAATACTTC
11581	0.975	MNL1	GAGG
11586	0.976	ALU1	AGCT
11591	0.976	MNL1	GAGG
11631	0.980	DDE1	CTTAG
11648	0.981	XBA1	TCTAGA
11652	0.981	MNL1	GAGG
11701	0.985	MBO11	GAAGA
11765	0.991	ALU1	AGCT
11778	0.992	ALU1	AGCT
11828	0.996	HIND111	AAGCTT
11829	0.996	ALU1	AGCT
11845	0.998	BAMH1	GGATCC
11846	0.998	MBO1	GATC
11868	0.999	PVU11	CAGCTG
11869	1.000	ALU1	AGCT

FIG. 9(a)

```
                   -40                                            -20
                    M I M A E S P G L I T I C L L G Y L L S A E C T|V F L D H E N A N K
TTTGCTAGCAGATTGTGAACATGATCATGGCAGAATCACCAGGCCTCATCACCATCTGCCTTTTAGGATATCTACTCAGTGCTGAATGTACAG|TTTTTCTTGATCATGAAAACGCCAACA
        10        20        30        40        50        60        70        80        90   t 100 ->   110       120

     -4    -1 1                                  20
             *
 I L N R F K R Y N S G K L E E F V Q G N L E R E C M E E K C S F E E A R E V F E
AAATTCTGAATCGGCCAAAGAGGTATAATTCAGGTAAATTGGAAGAGTTTGTTCAAGGGAACCTTGAGAGAGAATGTATGGAAGAAAAGTGTAGTTTTGAAGAAGCACGAGAAGTTTTTG
       130       140       150       160       170       180       190       200       210       220       230       240

           40                                    60
 N T E R T|T E F W K Q Y V|C G D Q C E S N P C L N G G S C K D D I N S Y E C W C
AAAACACTGAAAGAACA|ACTGAATTTTGGAAGCAGTATGTTG|CTGGAGATCAGTGTGAGTCCAATCCATGTTTAAATGGCGGCAGTTGCAAGGATGACATTAATTCCTATGAATGTTGGT
       250       260       270       280       290       300       310       320       330       340       350       360
     <- t | u ->           <- u | v ->

                80                                    100
 P F G F E G K N C E L|D V T C N I K N G R C E Q F C K N S A D N K V V C S C T E
GTCCCTTTCGATTTGAAGGAAAGAACTGTGAATTAG|ATGTAACATGTAACATTAAGAATGGCAGATGCGAGCAGTTTTGTAAAAATAGTGCTGATAACAAGGTGGTTTGCTCCTGTACTG
       370       380       390       400       410       420       430       440       450       460       470       480
                        <- v | w ->

           120                                   140
 G Y R L A E N Q K S C E P A|V P F P C G R V S V S Q T S K L T R A E A V F P D V
AGGGATATCGACTTGCAGAAAACCAGAAGTCCTGTGAACCAGCAG|TGCCATTTCCATGTGGAAGAGTTTCTGTTTCACAAACTTCTAAGCTCACCCGTGCTGAGGCTGTTTTTCCTGATG
       490       500       510       520       530       540       550       560       570       580       590       600
                              <- w | x ->

           160                                   180
 L Y V N S T E A E T I L D N I T Q S T Q S F N D F T R V V G G E D A K P G Q F P
TGGACTATGTAAATTCTACTGAAGCTGAAACCATTTTGGATAACATCACTCAAAGCACCCAATCATTTAATGACTTCACTCGGGTTGTTGGTGGAGAAGATGCCAAACCAGGTCAATTCC
       610       620       630       640       650       660       670       680       690       700       710       720

           200                                   220
 W Q|V V L N G K V D A F C G G S I V N E K W I V T A A H C V E T G V K I T V V A
CTTGGCAG|GTTGTTTTGAATGGTAAAGTTGATGCATTCTGTGGAGGCTCTATCGTTAATGAAAAATGGATTGTAACTGCTGCCCACTGTGTTGAAACTGGTGTTAAAATTACAGTTGTCG
       730       740       750       760       770       780       790       800       810       820       830       840
 <- x | y ->

           240                                   260
 |G E H N I E E T E H T E Q K R N V I R I I P H H N Y N A A I N K Y N H D I A L L
CAG|GTGAACATAATATTGAGGAGACAGAACATACAGAGCAAAAGCGAAATGTGATTCGAATTATTCCTCACCACAACTACAATGCAGCTATTAATAAGTACAACCATGACATTGCCCTTC
       850       860       870       880       890       900       910       920       930       940       950       960
 <- y | z ------>

           280                                   300
 E L D E P L V L N S Y V T P I C I A D K E Y T N I F L K F G S G Y V S G W G R V
TGGAACTGGACGAACCCTTAGTGCTAAACAGCTACGTTACACCTATTTGCATTGCTGACAAGGAATACACGAACATCTTCCTCAAATTTGGATCTGGCTATGTAAGTGGCTGGGGAAGAG
       970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
```

FIG. 9(b)

```
                  320                                                                          340
 F  H  K  G  R  S  A  L  V  L  Q  Y  L  R  V  P  L  V  D  R  A  T  C  L  R  S  T  K  F  T  I  Y  N  N  M  F  C  A  G  F
TCTTCCACAAAGGGAGATCAGCTTTAGTTCTTCAGTACCTTAGAGTTCCACTTGTTGACCGAGCCACATGTCTTCGATCTACAAAGTTCACCATCTATAACAACATGTTCTGTGCTGGCT
      1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200

                  360                                                                          380
 M  E  G  G  R  D  S  C  Q  G  D  S  G  G  P  H  V  T  E  V  E  G  T  S  F  L  T  G  I  I  S  W  G  E  E  C  A  M  K  G
TCCATGAAGGAGCTAGAGATTCATGTCAAGGAGATAGTGGGGGACCCCATGTTACTGAAGTGGAAGGGACCAGTTTCTTAACTGGAATTATTAGCTGGGGTGAAGAGTGTGCAATGAAAG
      1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320

                  400                                            415
 K  Y  G  I  Y  T  K  V  S  R  Y  V  N  W  I  K  E  K  T  K  L  T  *
GCAAATATGGAATATATACCAAGGTATCCCGGTATGTCAACTGGATTAAGGAAAAAACAAAGCTCACTTAATGAAAGATGGATTTCCAAGGTTAATTCATTGGAATTGAAAATTAACAGG
      1330      1340      1350      1360      1370      1380      1390      1400      1410      1420      1430      1440

GCCTCTCACTAACTAATCACTTTCCCATCTTTTGTTAGATTTGAATATATACATTCTATGATCATTGCTTTTTCTCTTTACAGGGGAGAATTTCATATTTACCTGAGCAAATTGATTAG
      1450      1460      1470      1480      1490      1500      1510      1520      1530      1540      1550      1560

AAAATGGAACCACTAGAGGAATATAATGTGTTAGGAAATTACAGTCATTTCTAAGGGCCCAGCCCTTGACAAAATTGTGAAGTTAAATTCTCCACTCTGTCCATCAGATACTATGGTTCT
      1570      1580      1590      1600      1610      1620      1630      1640      1650      1660      1670      1680

CCACTATGGCAACTAACTCACTCAATTTTCCCTCCTTAGCAGCATTCCATCTTCCCGATCTTCTTTGCTTCTCCAACCAAAACATCAATGTTTATTAGTTCTGTATACAGTACAGGATCT
      1690      1700      1710      1720      1730      1740      1750      1760      1770      1780      1790      1800

TTGGTCTACTCTATCACAAGGCCAGTACCACACTCATGAAGAAAGAACACAGGAGTAGCTGAGAGGCTAAAACTCATCAAAAACACTACTCCTTTTCCTCTACCCTATTCCTCAATCTTT
      1810      1820      1830      1840      1850      1860      1870      1880      1890      1900      1910      1920

TACCTTTTCCAAATCCCAATCCCCAAATCAGTTTTTCTCTTTCTTACTCCCTCTCTCCCTTTTACCCTCCATGGTCGTTAAAGGAGAGATGGGGAGCATCATTCTGTTATACTTCTGTAC
      1930      1940      1950      1960      1970      1980      1990      2000      2010      2020      2030      2040

ACAGTTATACATGTCTATCAAACCCAGACTTGCTTCCATAGTGGGGACTTGCTTTTCAGAACATAGGGATGAAGTAAGGTGCCTGAAAAGTTTGGGGCAAAAGTTTCTTTCAGAGAGTTA
      2050      2060      2070      2080      2090      2100      2110      2120      2130      2140      2150      2160
```

FIG. 9(c)

```
AGTTATTTTATATATATAATATATATATAAAATATATAATATACAATATAAATATATAGTGTGTGTGTGTATGCGTGTGTAGACACACACGCATACACACATATAATGGAAGCAATAA
         2170      2180      2190      2200      2210      2220      2230      2240      2250      2260      2270      2280

GCCATTCTAAGAGCTTGTATGCTTATGGAGGTCTGACTAGGCATGATTTGACGAAGGCAAGATTGGCATATCATTGTAACTAAAAAAGCTGACATTGACCCAGACATATTGTACTCTTTC
         2290      2300      2310      2320      2330      2340      2350      2360      2370      2380      2390      2400

TAAAAATAATAATAATAATGCTAACAGAAAGAAGAGAACCGTTCGTTTGCAATCTACAGCTAGTAGAGACTTTGAGGAAGAATTCAACAGTGTGTCTTCAGCAGTGTTCAGAGCCAAGCA
         2410      2420      2430      2440      2450      2460      2470      2480      2490      2500      2510      2520

AGAAGTTGAAGTTGCCTAGACCAGAGGACATAAGTATCATGTCTCCTTTAACTAGCATACCCCGAAGTGGAGAAGGGTGCAGCAGGCTCAAAGGCATAAGTCATTCCAATCAGCCAACTA
         2530      2540      2550      2560      2570      2580      2590      2600      2610      2620      2630      2640

AGTTGTCCTTTTCTGGTTTCGTGTTCACCATGGAACATTTTGATTATAGTTAATCCTTCTATCTTGAATCTTCTAGAGAGTTGCTGACCAACTGACGTATGTTTCCCTTTGTGAATTAAT
         2650      2660      2670      2680      2690      2700      2710      2720      2730      2740      2750      2760

AAACTGGTGTTCTGGTTCAAA
         2770
```

```
                              BamHI  PvuII       HindIII

Oligo N3              5'       GATCCAGCTGA        3'
                                   .......
                                   .......
Oligo N4                3'       GTCGACTTCGA        5'
```

Fig. 10

```
Eco RI                                        ClaI   HindIII  PvuII  BamHI
                  10          20                  30         40         50
5'  GAA TTCTCATGTT TGACAGCTTA TCATCGATAA GCTTCAGCTG GATCCTCTAC
              60
    GCCGGACGCA    3'
```

Fig. 11

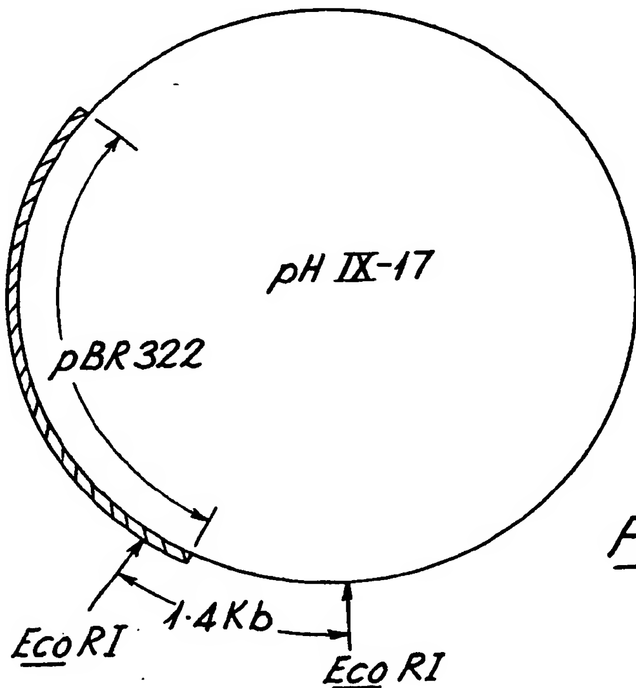
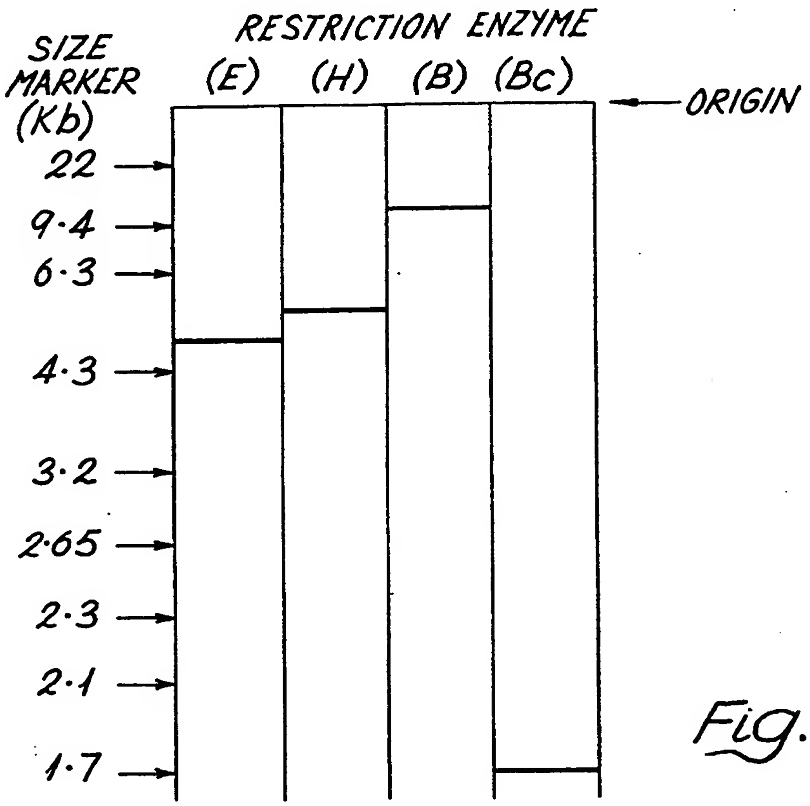


Fig. 12



RESTRICTION ENZYME
SIZE MARKER (Kb)
(E)
(H)
(B)
(Bc)
ORIGIN
22
9·4
6·3
4·3
3·2
2·65
2·3
2·1
1·7

Fig. 13

SPECIFICATION
Genetic engineering

BACKGROUND OF THE INVENTION
1. *Field of the invention*
This invention is in the field of genetic engineering relating to factor IX DNA.

2. *Description of prior art*
Factor IX (Christmas factor or antihaemophilic factor B) is the zymogen of a serine protease which is required for blood coagulation via the intrinsic pathway of clotting (Jackson & Nemerson, Ann.Rev.Biochem. *49*, 765—811, 1980). This factor is synthesised in the liver and requires vitamin K for its biosynthesis (Di Scipio & Davie, Biochem. *18*, 899—904, 1979).

Human factor IX has been purified and characterised, but details of the amino acid sequence are fragmentary. It is a single-chain glycoprotein, with a molecular weight of approximately 60,000 (Suomela, Eur.J.Biochem. *71*, 145—154, 1976). Like other vitamin K-dependent plasma proteins, human factor IX contains in the amino-terminal region approximately 12 gamma-carboxyglutamic acid residues (Di Scipio & Davie, Biochem. *18*, 899—904, 1979)

During the clotting process, and in the presence of Ca^{++} ions, factor IX is acted upon by activated factor IX (IXa) by the cleavage of two internal peptide bonds, releasing an activation glycopeptide of 10,000 daltons (Di Scipio *et al.*, J.Clin. Invest. *61*, 1528—1538, 1978). The activated factor IX (IXa) is composed of two chains held together by at least one disulphide bond. Factor IXa then participates in the next step in the coagulation cascade by acting on factor X in the presence of activated factor VIII, Ca^{++} ions, and phospholipids (Lindquist *et al.*, J.Biol.Chem. *253*, 1902—1909, 1978).

Individuals deficient in factor IX (Christmas disease or haemophilia B) show bleeding symptoms which persist throughout life. Bleeding may occur spontaneously or following injury. This may take place virtually anywhere. Bleeding into the joints is common, and after repeated haemorrhages, may result in permanent and crippling deformities. The condition is a sex-linked disorder affecting males. Its frequency in the population is approximately 1 in 30,000 males.

The current method of diagnosing Christmas disease involves measurement of the titre of factor IX in plasma by a combination of a clotting assay and in immunochemical assay. Treatment of haemorrhage in the disease consists of factor IX replacement by means of intravenous transfusion of human plasma protein concentrates enriched in factor IX. The enrichment of plasma in factor IX is a time-consuming process.

Summary of the invention
After considerable research and experiment, important progress has now been made towards producing artificial human factor IX by recombinant DNA technology (genetic engineering). Thus, the cloning of DNA sequences which are substantially the same as extensive sequences occurring in the human factor IX genome has been achieved.

The invention arises from the finding that an extensive DNA sequence of the human factor IX genome can be obtained by a clever and laborious combination of chemical synthesis and artificial biosynthesis, starting from elementary nucleotide or dinucleotide "building blocks", as will be described below.

A major feature of the invention comprises recombinant DNA which comprises a cloning vehicle DNA sequence and a sequence foreign thereto (i.e. foreign to the vehicle) which is substantially the same as a sequence occurring in the human factor IX genome. A 11873 nucleotide long part of such a foreign sequence has been identified and a very large part of it has been sequenced by the Maxam-Gilbert sequencing method. A 129 nucleotide length of this sequence is more than sufficient to characterise it unambiguously as coding for a specific protein and a particular such length is regarded herein as useful to characterise the whole sequence inserted in the cloning vehicle as one occurring in the human factor IX genome. Other cloned sequences can then be verified as belonging to the human factor IX genome by determining that part thereof is identical to a region of the first-mentioned sequence, i.e. the sequences have a common identity in an overlapping region.

A further feature of the invention therefore comprises recombinant DNA which comprises a cloning vehicle or vector DNA sequence and a DNA sequence foreign thereto which consists of or includes substantially the following sequence of 129 nucleotides (which should be read in rows of 30 across the page):—

ATGTAACATG TAACATTAAG AATGGCAGAT

GCGAGCAGTT TTGTAAAAAT AGTGCTGATA

ACAAGGTGGT TTGCTCCTGT ACTGAGGGAT

ATCGACTTGC AGAAAACCAG AAGTCCTGTG

AACCAGCAG (1)

The invention includes particularly recombinant DNA which comprises a cloning vehicle DNA sequence and a sequence foreign to the cloning vehicle, wherein the foreign sequence includes substantially the whole of an exon sequence of the human factor IX genome. The 129-nucleotide sequence described above corresponds substantially to such an exon sequence. Another such exon sequence which independently characterises the human factor IX DNA is the 203-nucleotide sequence substantially as follows (again reading in rows of 30 across the page):—

TGCCATTTCC ATGTGGAAGA GTTTCTGTTT

CACAAACTTC TAAGCTCACC CGTGCTGAGG

CTGTTTTTCC TGATGTGGAC TATGTAAATT

CTACTGAAGC TGAAACCATT TTGGATAACA

TCACTCAAAG CACCCAATCA TTTAATGACT

TCACTCGGGT TGTTGGTGGA GAAGATGCCA

AACCAGGTCA ATTCCCTTGG CAG

The intron sequences of the human factor IX genome are excised during the transcription process by which mRNA is made in human cells. Only exon sequences are translated into protein. DNA coding for factor IX has been prepared from human mRNA. This cDNA has been partly sequenced and found to contain the same 129- and 203-nucleotide sequences set out above.

The invention also includes recombinant DNA which comprises a cloning vehicle sequence and a DNA sequence foreign to the cloning vehicle, wherein the foreign sequence comprises a DNA sequence which is complementary to human factor IX mRNA. Such a recombinant cDNA can be isolated from a library of recombinant cDNA clones derived from human liver mRNA by using an exon of the genomic human factor IX DNA (or part thereof) as a probe to screen this library and thence isolating the resulting clones.

The invention also includes recombinant DNA in which the foreign sequence is any fragment of human factor IX DNA, particularly of length at least 50 and preferably at least 75 nucleotides or base-pairs. It includes such recombinant DNA whether or not part of the 129 or 203-base-pair sequence defined above. It includes especially part or all of the exon sequences of human factor IX genomic DNA. Various short lengths up to about 11 kilobases (11,000 nucleotides or base-pairs) long have been prepared by use of various restriction endonucleases. Methods of isolating recombinant DNA from clones are well known and some are described hereinafter. The DNA of the invention can be single or double stranded form.

The recombinant human factor IX DNA of this invention is useful as a tool of recombinant DNA technology. Thus it is useful as the first stage in the production of artificial human factor IX and in the preparation of probes for diagnostic purposes.

In the production of the artificial human factor IX it is contemplated that appropriate cDNA or genomic clones will be introduced into a suitable expression vector in either mammalian or bacterial systems. For mammalian studies, the gene might be too long to be conveniently retained in one clone. Therefore a suitable artificial "minigene" will be designed and constructed from suitable parts of the cDNA and genomic clones. The minigene will be under the control of its own promoter or instead will be replaced by an artificial one, perhaps the mouse metallothioneine I promoter. The resultant 'minigene' will then be introduced into mammalian tissue culture cells e.g. a hepatoma cell line, and selection for clones of cells synthesising maximum amounts of biologically active factor IX will be carried out. Alternatively "genetic farming" could be employed as has been demonstrated for mouse growth hormone (Palmiter *et al*, Nature *300*, 611—615, 1982). The minigene would be micro-injected into the pronucleus of fertilised eggs, followed by *in vivo* cloning and selection for progeny producing the largest quantity of human factor IX in blood. Alternatively, it is contemplated that the cDNA clone or selected parts of it will be linked to a suitable strong bacterial promotor, e.g. a *Lac* or *Trp* promotor or the lamdba P_R or P_L, and a factor IX polypeptide obtained therefrom.

The natural factor IX polypeptide is synthesised as a precursor containing both a signal and propeptide region. They are both normally cleaved off in the production of the definitive length protein. Even this product is merely a precursor. It is biologically inactive and must be gamma-carboxylated at 12 specific N-terminal glutamic acid residues in the so called 'GLA' domain by the action of a specific vitamin K-dependent carboxylase. In addition, two carbohydrate molecules are added to the connecting peptide region of the molecule, but is remains unknown whether they are required for activity. The substrate for the carboxylase is unknown and could be the precursor factor IX polypeptide or alternatively the definitive length protein. Therefore various relevant polypeptides both with and without the precursor domains will be "constructed" using genetic engineering methods in bacterial hosts. They will then be tested as substrates for the conversion of inactive to biologically active factor IV *in vitro* by the action of partially purified preparations of the carboxylase enzyme which can be isolated from liver microsomes or other suitable sources.

For diagnostic purposes, the recombinant human genomic factor IX DNA or recombinant human mRNA-derived factor IX DNA has a wide variety of uses. It can be cleaved by enzymes or combinations of two or more enzymes into shorter fragments of DNA which can be recombined into the cloning vehicle, producing "sub-clones". These sub-clones can themselves be cleaved by restriction enzymes to DNA molecules suitable for preparing probes. A probe DNA (by definition) is labelled in some way, conveniently radiolabelled, and can be used to examine in detail mutations in the human DNA which ordinarily would produce factor IX. Several different probes have been produced for examining several different regions of the genome where mutation was suspected to have occurred in patients. Failure to obtain hybridisation from such a probe indicates that the sequence of the probe differs in the patient's DNA. In particular it has been shown that Christmas disease can be detected or confirmed by such methodology. Useful probes can contain intron and/or exon regions of the genomic DNA or can

contain cDNA derived from the mRNA.

The invention includes particularly probe DNA, i.e. which is labelled, and of a length suitable for the probing use envisaged. It can be single-stranded or double-stranded over at least the human factor IX DNA probing sequences thereof and such sequences will usually have a length of at least 15 nucleotides, preferably at least 19—30 nucleotides in order to have a reasonable probability of being unique They will not usually be larger than 5 kb and rarely longer than 10 kb.

The invention accordingly includes a DNA molecule, comprising part of the human factor IX DNA sequence, whether or not labelled, whether intron or exon or partly both. It also includes human cDNA corresponding to part of all of human factor IX mRNA. It includes particularly a solution of any DNA of the invention, which is a form in which it is conveniently obtainable by electroelution from a gel.

The invention includes, of course, a host transformed with any of the recombinant DNA of the invention. The host can be a bacterium, for example an appropriate strain of *E.coli*, chosen according to the nature of the cloning vehicle employed. Useful hosts may include strains of *Pseudomonas*, *Bacillus subtilis* and *Bacillus stearothermophilus*, other *Bacilli*, yeasts and other fungi and mammalian (including human) cells.

One process practised in connection with this invention for preparing a host transformed with the recombinant DNA of the invention is based on the following steps:—

(1) synthesising an oligodeoxynucleotide having a nucleotide sequence comprising that occurring in bovine factor IX messenger RNA coding for amino acids 70—75 or 348—352 of bovine factor IX, and labelling the oligodeoxynucleotide to form a probe;

(2) preparing complementary DNA to a mixture of bovine mRNAs;

(3) inserting the complementary DNA in a cloning vector to form a mixture of recombinant bovine cDNAs;

(4) transforming a host with said mixture of recombinant bovine cDNAs to form a library of clones and multiplying said clones;

(5) probing the clones with the synthetic oligodeoxynucleotide probe obtained in step 1 and isolating the resultant recombinant bovine factor IX cDNA-containing clone;

(6) digesting the recombinant bovine factor IX cDNA from said clone with one or more enzymes to produce a bovine factor IX cDNA molecule comprising a shorter sequence of bovine factor IX DNA, but preferably at least 50 base-pairs long; and

(7) probing a library of recombinant human genomic DNA in a transformed host with the shorter sequence bovine factor IX cDNA molecule, to hybridise the human genomic DNA to the said recombinant bovine factor IX DNA and isolating the resultant recombinant DNA-transformed host.

Brief description of the drawings

Figure 1 shows the structure of a published amino-acid sequence of bovine factor IX polypeptide, the deduced sequence of the mRNA from which it would be translated and the structures of oligonucleotides (oligo-N1 and N2) synthesised in the course of this invention;

Figures 2 and 3 show the chemical formulae of "building blocks" used to synthesise the oligonucleotides referred to in Figures 1 and 11;

Figure 4 is an elevational view, partly sectioned, showing an apparatus for synthesising oligonucleotides;

Figure 5 shows the sequence of part of the bovine factor IX cDNA obtained in this invention;

Figure 6 is a map showing the organisation of an approximately 27 kb length of human factor IX genomic DNA and is divided into five portions, showing:—

(a) the exon regions;

(b) the 11,873- nucleotide length sequenced;

(c) cDNA molecules obtained by restriction with various endonucleases, sub-cloned and subsequently used as probes;

(d) DNA molecules obtained by restriction with various endonucleases; and

(e) three regions of human factor IX genomic DNA derived from three clones in lambda phage vector.

Figure 7 shows the sequence of the DNA of Figure 6(b) and in parts the encoded protein;

Figure 8 shows a restriction enzyme chart of the sequence shown in Figure 7;

Figure 9 shows part of the sequence of the human factor IX cDNA and its encoded protein;

Figure 10 shows the structure of a pair of complementary oligonucleotides (oligo N3 and N4) synthesised in the course of this invention;

Figure 11 shows part of the DNA sequence of the vector pAT153/PvuII/8 of this invention, in the region where it differs from pAT153;

Figure 12 is a diagram of plasmid pHIX17 of the invention showing the origin of the 1.4 kb fragment used for probing and initial sequencing; and

Figure 13 shows the position of the major radioactive bands on probing a "Southern blot" of normal human DNA, cut by the restriction enzymes *Eco*RI(E), *Hind*III(H), *Bgl*II(B) and *Bcl*I(Bc), with a sub-clone of the recombinant human factor IX DNA of this invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

1. *General description*

A recombinant DNA of the invention can be extracted by means of probes from a library of cloned human genomic DNA. This is a known recombinant library and the invention does not, of course, extend to human genomic factor IX DNA when present in such a library. The probes used were of bovine factor IX cDNA (DNA complementary to bovine mRNA), which were

prepared by an elaborate process involving firstly the preparation of recombinant bovine cDNA from a bovine mRNA starting material, secondly the chemical syntheses of oligonucleotides, thirdly their use to probe the recombinant bovine cDNA, in order to extract bovine factor IX cDNA and fourthly the preparation of suitable probes of shorter length from the recombinant bovine factor IX cDNA. The first probe tried appeared to contain an irrelevant sequence and the second probe tried not containing it, proved successful in enabling a single clone of the human genomic factor IX DNA to be isolated. This clone is designated lambda HIX—1. The steps involved are described in more detail in the sub-section "Examples" appearing hereinafter, and the second probe comprises the 247 base-pair DNA sequence of bovine factor IX cDNA indicated in Figure 5 of the drawings. The invention therefore provides specifically a recombinant DNA which comprises a cloning vehicle sequence and a DNA sequence foreign to the cloning vehicle, which recombinant DNA hybridises to a 247 base-pair sequence of bovine factor IX cDNA indicated in Figure 5 (by the arrows at each end thereof).

The cloning vehicle or vector employed in the invention can be any of those known in the genetic engineering art (but will be chosen to be compatible with the host). They include *E.coli.* plasmids, e.g. pBR322, pAT153 and modifications thereof, plasmids with wider host ranges, e.g. RP4 plasmids specific to other bacterial hosts, phages, especially lambda phage, and cosmids. A cosmid cloning vehicle containing a fragment of phage DNA including its cos (cohesive-end site) inserted in a plasmid. The resultant recombinant DNA is circular and has the capacity to accommodate very large fragments of additional foreign DNA.

Fragments of human factor IX genomic DNA can be prepared by digesting the cloned DNA with various restriction enzymes. If desired, the fragments can be religated to a cloning vehicle to prepare further recombinant DNA and thereby obtain "sub-clones". In connection with this embodiment a new cloning vehicle has been prepared. This is a modified pAT153 plasmid prepared by ligating a *Bam*HI and *Hind*III double digest of pAT153 to a pair of complementary double sticky-ended oligonucleotides having a DNA sequence providing a *Bam*HI restriction residue at one end, a *Hind*III restriction residue at the other end and a *Pvu*II restriction site in between.

While the invention is described herein with reference to human genomic factor IX DNA in particular, the invention includes human factor IX cDNA (complementary to human factor IX mRNA) which contains substantially the same sequences. A library of human cDNA has been prepared and probed with human factor IX genomic DNA to isolate human factor IX cDNA from the library. For this purpose the probe DNA is conveniently of relatively short length and must include at least one exon sequence. The invention therefore includes a process of preparing a host transformed with recombinant DNA, comprising cloning vector sequences and a sequence of nucleotides comprised in cDNA complementary to human factor IX mRNA, which process comprises probing a library of clones containing recombinant DNA complementary to human mRNA with a probe comprising a labelled DNA comprising a sequence complementary to part or all of an exon region of the human factor IX genome.

2. *Examples*

A. Bacteria used

E.coli K—12 strain MC 1061 (Casadaban & Cohen, J.Mol.Biol. *138*, 179—207, 1980), *E.coli* K—12 strain HB 101 (Boyer & Roulland-Dussoix, J.Mol.Biol *41*, 459—472, 1969) and *E.coli* K—12 strain K803 which is a known strain used by genetic engineers.

B. Source and purification of bovine factor IX, anti-bovine factor IX antibody, and bovine mRNA

Highly purified bovine factor IX and rabbit anti-bovine factor IX antiserum were gifts from Dr. M. P. Esnouf. Analysis of the purified bovine factor IX on a denaturating polyacrylamide gel showed that it has a purity of greater than 99%. Specific anti-factor IX immunoglobulins used for immunoprecipitation experiments were purified as described by Choo *et al.*, Biochem.J. *199*, 527—535, 1981, by passage of the crude antiserum through a Sepharose—4B column onto which pure bovine factor IX has been coupled.

Bovine mRNA was obtained from calf liver and isolated by the guanidine hydrochloride method (Chirgwin *et al.*, Biochem. *18*, 5294—5299, 1979). The mRNA preparation was passaged through an oligo dT-cellulose column (Caton and Robertson, Nucl. Acids Res. *7*, 1445—1456, 1979) to isolate poly(A) + mRNA.
Poly(A) + mRNA was translated in a rabbit reticulocyte cell-free system in the presence of ^{35}S-cysteine as described by Pelham and Jackson (Eur. J.Biochem. *67*, 247—256, 1976). At the end of the translation reaction, factor IX polypeptide was precipitated by the addition of specific anti-factor IX immunoglobulins. The immunoprecipitation procedure was as described by Choo *et al.*, Biochem.J. *181*, 285—294, 1979. The immunoprecipitated material was washed throughly and resolved on a two-dimensional SDS-polyacrylamide gel (Choo *et al.*, Biochem.J. *181*, 285—294, 1979), by isoelectric focussing in one dimension and electrophoresis in another. Some polypeptides of known molecular weight were subjected to this procedure, to serve as reference points. The immunoprecipitated material showed 4 pronounced spots, all in the 50,000 molecular weight region and with separated isoelectric points. These predominant spots of molecular weight about 50,000 represent a single polypeptide chain plus a possible prepeptide signal sequence, a deduction compatible with published data (Katayama *et al.*, Proc. Natl.Acad. Sci.USA *76*, 4990—4994, 1979).

When the gel analysis was repeated for the

same material but immunoprecipitated in the presence of unlabelled pure bovine factor IX, the 4 spots appeared at reduced intensity, indicating that the translation product is specifically competed for by pure factor IX. Thirdly, immunoprecipitation was performed using a control rabbit antiserum, i.e. from a rabbit which had not been immunised with factor IX. None of the 4 spots appeared. These results therefore indicate that the translation product was a factor IX polypeptide.

The specific immunological/cell-free translation assay established above was used to monitor the enrichment of factor IX mRNA on sucrose gradient centrifugations. Total poly(A) + mRNA was resolved by two successive separations by sucrose gradient centrifugations. When individual fractions from the gradient were assayed by the above method, a fraction of size 20—22 Svedberg units (approx. 2.5 kilobases of RNA) region was found to be enriched (approx. ten-fold) for the bovine factor IX mRNA. This enriched fraction was used in the subsequent cloning experiments.

C. Synthesis of specific bovine factor IX deoxyoligonucleotide mixtures

Starting from a knowledge of the amino acid sequence of bovine factor IX (Katayama *et al.*, Proc.Natl.Acad.Sci. USA *76*, 4990—4994, 1979), the synthesis of two mixtures of oligonucleotide probes was designed. These probes consisted of DNA sequences coding for two different regions of the protein. The regions selected were those known to differ in sequence in the analogous serine proteases, prothrombin, Factor C and Factors VII and X and were those corresponding to amino acids 70—75 and 348—352 respectively. The 70—75 region was particularly favourable in that the mixture of oligonucleotides synthesised, i.e. oligo N2A and oligo N2B, contained all 16 possible sequences that might occur in a 17 nucleotide long region of the mRNA corresponding to amino acids 70—75. The oligo N2A—N2B mixture is hereinafter called "oligo N2" for brevity.

Figure 1 of the drawings shows the two selected regions of the known amino acid sequence of bovine factor IX, the corresponding mRNA and the oligonucleotides synthesised. Since some of the amino acids are coded for by more than one nucleotide triplet, there are 4 ambiguities in the mRNA sequence shown for amino acids 70—75 and therefore 16 possible individual sequences.

The nucleotide mixtures oligo N1 and oligo N2 were synthesized using the solid phase phosphotriester method of Duckworth *et al.*, Nucl.Acids Res. *9*, 1691—1706, 1981, modified in two ways. Firstly, *o*-chlorophenyl rather than *p*-chlorophenyl blocking groups were used for the phosphotriester grouping, and were incorporated in the mononucleotide and dinucleotide "building blocks". Figures 2 and 3 of the drawings show (a) dinucleotide and (b) mononucleotide "building blocks". DMT = 4,4' - dimethoxytrityl and B = 6-N-benzoyl-adenin-9-yl, 4-N-benzoylcytosin-1-yl, 2-N-isobutyrylguanin-9-yl or thymin-1-yl, depending on the nucleotide selected. Secondly, the "reaction cell" used for the successive addition of mono- or dinucleotide "building blocks" was miniaturised so that the coupling step with the condensing agent 1-(mesitylene-2-sulphonyl)-3-nitro-1,2,4-triazole (MSNT) was carried out in a volume of 0.5ml pyridine containing 3.5 micromoles of polydimethylacrylamide resin, 17.5 micromoles of incoming dinucleotide (or 35 micromoles of mononucleotide) and 210 micromoles of MSNT.

Figure 4 of the drawings is an elevational view of the microreaction cell 1 and stopper 2 used for oligonucleotide synthesis, drawn 70% of actual size. The device comprises a glass-to-PTFE tubing joint 3 at the inlet end of the stopper 2. The stopper has an internal conduit 4 which at its lower end passes into a hollow tapered ground glass male member 5 and thence into a sintered glass outlet 6 to the stopper. The cell 1 has a ground glass female member 7 complementary to the member 5 of the stopper, leading to reaction chamber 8, the lower end of which terminates in a sintered glass outlet 9. This communicates with glass tubing 10 and a 1.2mm. "Interflow" tap 11. Further glass tubing 10, beyond the tap 11, leads to the outlet glass-to-PTFE tubing joint 12. Pairs of ears 13 on the stopper and cell enable them to be joined together by springs (not shown) in a liquid-tight manner.

After completion of the synthesis and deprotection, fractionation was carried out by high pressure liquid chromatography (Duckworth *et al.*, see above) and the peak tubes corresponding to the product of correct chain length were located by labelling of fractions at their 5'-hydroxyl ends using [gamma-^{32}p]-ATP and T4 polynucleotide kinase, followed by 20% 7M urea polyacrylamide gel electrophoresis. The position on the gel of the 17- and 14- oligonucleotides was determined by separately labelling, by the method described above, 17- and 14- nucleotide long "marker" oligonucleotides and subjecting these to the same gel electrophoresis.

D. Preparation of libraries of cDNA sequences for bovine mRNA

Two different approaches were used for the generation of cloned cDNA library:—

(i) *MboI library* First strand cDNA was synthesised using the sucrose gradient-enriched poly(A)+bovine mRNA as template. The conditions used were as described by Huddleston & Brownlee, Nucl. Acids Res.*10*, 1029—1030, 1981, except that 2 micrograms of oligo N—1, 20—30 micrograms of the mRNA, 10 microcuries [alpha-^{32}P]-dATP (Amersham, 3000 Ci/mmole), and 50 U of reverse transcriptase were used in a 50 microlitre reaction. "dNTP" in Figure 1 denotes the mixture of 4 deoxynucleoside triphosphates required for synthesis. Oligo N—1 hybridises to the corresponding region on the mRNA (refer to Figure 1) and thereby acts as a primer for the

initiation of transcription. It was used in order to achieve a further enrichment for factor IX mRNA. At the end of the cDNA synthesis reaction, the cDNA was extracted with phenol and desalted on a Sephadex-G100 column, before it was treated with alkali (0.1 M NaOH, 1mM EDTA) for 30 min. at 60°C to remove the mRNA strand. Second strand DNA synthesis was then carried out exactly as published (Huddleston & Brownlee, Nucl.Acids Res. *10*, 1029—1038, 1981).

The double-stranded DNA was next cleaved with the restriction enzyme *MboI* and ligated to the plasmid vector pBR322 which had been cut with *Bam*HI and treated with calf intestinal alkaline phosphatase to minimise vector self-religation. Phosphatase treatment was carried out by incubating 5 micrograms of *Bam*HI-cut pBR 322 plasmid with 0.5 microgram calf intestinal phosphatase (Boehringer; in 10mM Tris — HCl buffer, pH 8.0) in a volume of 50 microlitres at 37°C for 10 minutes, see Huddleston & Brownlee *supra*.

The ligated DNA was used to transform *E.coli* strain MC 1061. For transformation *E.coli* MC 1061 was grown to early exponential phase as indicated by an absorbancy of 0.2 at 600 nm and made "competent" by treating the pelleted bacterial cells first with one half volume, followed by repelleting, and then with 1/50 volume of the original growth medium of 100mM $CaCl_2$ 15% v/v glycerol and 10mM PIPES—NaOH, pH 6.6 at 0°C. Cells were immediately frozen in a dry ice/ethanol bath to −70°C. For transformation, 200 microlitre aliquots were mixed with 10 microlitres of the recombinant DNA and incubated at 0°C for 10 minutes followed by 37°C for 5 minutes. 200 microlitres of L-broth (bactotryptone 10g., yeast extract 5g., sodium chloride 10g., made up to 1 litre with deionised water) were then added and incubation continued for a further 30 minutes at 37°C. The solution was then plated on the appropriate antibiotic agar (see below). A library of about 7,000 ampicillin-resistant colonies was thus obtained. They were ampicillin-resistant because they contained the beta-lactamase gene of pBR 322. Of these, aprox. 85% were found to be tetracycline-sensitive.

(ii) *dC/dG tailed library* In the preparation of this library, first strand cDNA was synthesised as described for the above library except that oligo $dT_{(12-18)}$ was used as a primer to initiate cDNA synthesis. Following this, the cDNA was tailed with dCTP using terminal transferase and back-copied with the aid of oligo $dG_{(12-18)}$ primer and reverse transcriptase to give double stranded DNA, exactly according to the method of Land *et al.*, Nucl.Acids Res. *9*, 2251—2266, 1981. After a further tailing with dCTP, this material was annealed by hybridisation to a dGTP-tailed pBR322 plasmid at the *PstI* site. The hybrid DNA was used to transform *E.coli* strain MC 1061. A library of approximately 10,000 tetracycline-resistant colonies was obtained. Of these, approximately 80% were found to be sensitive to ampicillin, due to insertion of DNA into the ampicillin-resistant gene at the *PstI* site.

E. Isolation of specific bovine factor IX clones

(i) *From MboI library*

The library of colonies, in an unordered fashion, was transferred onto 13 Whatman 541 filter papers and amplified with chloramphenicol, to increase the number of copies of the plasmid in the colonies, as described by Gergen *et al.*, Nucl. Acids Res., *7*, 2115—2136 (1979). The filters were pre-hybridised at 65°C for 4h in 6 × NET (1×NET = 0.15m NaCl, 1mM EDTA, 15mM Tris-HCl, pH 7.5), 5 × Denhardt's, 0.5% NP40 non-ionic surfactant, and 1 microgram/ml. yeast RNA as described by Wallace *et al.*, Nucl. Acids Res. *9*, 879—894 (1981). Hybridisation was carried out at 47°C for 20h in the same solution containing 3×10^5cpm (0.7 nanogram/ml) of labelled oligo N—2 probe. Labelling was done by phosphorylation of the oligonucleotides at the 5' hydroxyl end using [gamma-^{32}P]-ATP and T4 phophokinase (Huddleston & Brownlee, Nucl.Acids Res. *10*, 1029—1038, 1981). At the end of the hybridisation, filters were washed successively at 0—4°C (2h), 25°C (10 min), 37°C (10 min) and 47°C (10 min). After radioautography of the filters from this screening, one colony showed a positive signal above background. This colony was designated BIX—1 clone.

(ii) *From dC/dG-tailed library*

Screening of this library, in an ordered array fashion, using oligo N—2 probe as described above has resulted in the identification of a positive clone. This was designated BIX—2 clone

F. Sequence characterisation of bovine factor IX cDNA clones

Characterisation of BIX—1 clone by restriction endonuclease cleavage indicated that it contained a DNA insert of about 430 base-pairs (data omitted, for brevity). Figure 5 shows part of the nucleotide sequence of the coding strand, determined by the Maxam-Gilbert method, extending over 304 nucleotides and provides direct evidence that it has the identity of a bovine factor IX sequence. Thus, nearly all of this 304 nucleotide sequence (corresponding to the amino acid residues 52—139) agrees with the nucleotide sequence predicted from the known bovine factor IX amino acid sequence data (Katayama *et al.*, Proc.Natl.Acad.Sci. *76*, 4990—4994, 1979). Over this region, there are no discrepancies between BIX—1 and these published data for factor IX, except at nucleotides 38—40 where the amino acid coded for is Asp instead of Thr. This amino acid change was similarly observed in a second, independent cDNA clone (BIX—2; see below). The remainder of the 304-nucleotide sequence, i.e. that shown in brackets in Figure 5, does not agree with the published bovine factor IX amino acid data of Katayama.

In Figure 5, the underlined portion denotes the sequence corresponding to the oligo N—2 probe

sequence, the asterisk denotes a nonsense codon, the brackets enclose a sequence which does not correspond to Katayama's amino acid data and the arrows indicate *Hin*fI restriction sites. The Katayama numbering system for amino acids is shown and this sequence is in the opposite orientation to the direction of transcription of the tetracycline-resistant gene of the plasmid.

By similar methods, BIX—2 clone was found to have a DNA insert of 102 nucleotides and this spans the nucleotide positions 7—108 as shown in Figure 5. The nucleotide sequences for BIX—1 and BIX—2 clones over this region (nucleotide 7—108) were identical.

G. Isolation of human factor IX gene

(i) *Initial clone — lambda HIX—1*

A library of cloned human genomic DNA, namely a *Hae*III/AluI lambda phage Charon 4A library prepared by Lawn *et al.*, Cell, *15*, 1157—1174, 1978, was used. 10^6 phage recombinants from this library were screened using the *in situ* plaque hybridisation procedure as described by T. Maniatis *et al.*, Cell, *15*, 687, 1978. Pre-hybridisation and hybridisation were carried out at 42°C in 50% formamide. After hybridisation, filters were washed at room temperature with 2 × SSC (1 × SSC = 0.15mM NaCl, 15mM sodium citrate, at pH 7.2) and 0.1% SDS, then at 65°C with 1 × SSC and 0.1% SDS.

Two DNA molecules, being restriction fragments from the factor IX cDNA cloned in BIX—1, were radiolabelled and used as probes in the hybridisation. The first fragment corresponds to nucleotide numbers −8 to 317 on the numbering system of Figure 5, and was isolated by *Sau*3AI digestion of BIX—1 plasmid DNA. The isolated DNA was labelled to high specific activity by incorporation of [alpha—^{32}P] -dATP using a nick translation (Rigby *et al.*, J. Mol.Biol. *113*, 237—251, 1977, modified, *vide infra*). Using this probe, 10 clones were isolated. These were plaque-purified and re-hybridised with a 247-nucleotide fragment from BIX—1 clone. This fragment, derived from nucleotides 3—249 can be seen from Figure 5. It contains only sequences in agreement with the Katayama bovine factor IX amino acid sequence and was isolated by *Hin*fI digestion of BIX—1 plasmid DNA. Only a single clone gave a positive hybridisation signal with this 247-nucleotide probe. This clone was further plaque-purified and the resulting clone was designated "lambda HIX—1".

(ii) *Subsequent genomic clones*

A sub-clone, pATIXcVII, of recombinant human factor IX cDNA from human liver mRNA, and prepared as described in Section L below, was linearised by digestion with *Hin*dIII and *Bam*HI. The resulting 2 kb cDNA molecule was purified by 1% agarose gel electrophoresis. After electroelution, about 100 ng of this cDNA was nick-translated with [alpha ^{32}p] dATP (see above) and used as a hybridisation probe to screen the *Hae*III/*Alu*I lambda phage Charon 4A human genomic DNA library for further genomic clones, using standard stringent hybridisation conditions. Two further human factor IX genomic clones, designated lambda HIX—2 and lambda HIX—3, were thus obtained.

H. Characterisation of human factor IX genomic clones

(i) *Restriction map*

The initial lambda HIX—1 clone was characterised by cleavage with various single and double digests with different restriction endonucleases and Southern blotting of fragments using the bovine factor IX cDNA probe (results omitted for brevity). The subsequently isolated lambda HIX—2 and 3 clones were characterised in the same way except that the human cDNA probe, pATIXcVII (see Section L below) was used for the Southern blots. From these results it emerged that the sequences in the factor IX genome corresponding to lambda HIX—2 and 3 overlapped with lambda HIX—1 as shown in Figure 6(e). In Section (d) of this Figure 6 are summarised the results of the analysis using the restriction enzymes *Eco*RI (E), *Hin*dIII (H), *Bg*lII (B), *Bam*HI (Ba) and *Pvu*II (P), and this serves as a restriction enzyme map.

(ii) *Sequencing*

Numerous sub-clones were isolated from a knowledge of the rectriction enzyme map as described in Section J(ii) below, the majority in a vector pAT153/PvuII/8. Examples of these sub-clones are shown in Figure 6(c) and a number were used and were of a convenient length for sequence analysis by the Maxam-Gilbert method (Maxam & Gilbert, Proc.Natl.Acad.Sci.USA *74*, 56—564, 1980).

Initially sequencing was done on part of a 1.4 kb *Eco*RI restriction fragment from the sub-clone pHIX—17, see below and J(i). A 403-nucleotide (base-pair) length was sequenced, of which a 129-nucleotide length was identified as lying within an exon region. This is the 129-nucleotide sequence used above to define the factor IX DNA.

Subsequently, a region of 11873 bases was sequenced in the central portion of the gene [see Figure 6(b)]. Figure 7 shows the sequence of one strand of the DNA. The nucleotides are arbitrarily numbered from 1 to 11873 in the 5′ to 3′ direction. The original 403-nucleotide sequence runs from Figure 7 nucleotides Nos. 4372 to 4774 and is indicated by O—O′. The 129-nucleotide sequence lying within the 403 one, runs from Figure 7 nucleotides Nos. 4442 to 4570 and is indicated by J—J′. This corresponds exactly to the "w" exon.

In detail, the sequence of nucleotides Nos. 1—7830 contains two short exons (nucleotides 4442—4570 and 7140—7342 respectively) marked w and x in Figure 6(a), J—J′ and J′—J″ in Figures 7 and 9. These code for amino acids 85—127, and 128—195 respectively of the amino acid sequence predicted from the human factor IX cDNA clone (Figure 9). There are no differences in amino acid sequences predicted from the genomic and cDNA clones of the

invention in these two exon regions. The sequence of the gene between residues 7831—11873 is less complete, containing several gaps, but is still a useful characterisation of the gene as it contains two "*Alu*I repeat" sequences, nucleotides 7960—8155 and 9671—9938. *Alu*I sequences are found in many genes. The repetition is not exact but there is a typical degree of homology between them. This further characterisation provides a useful cross-check on the accuracy of the restriction enzyme map. This emerges more clearly from the restriction enzyme chart of Figure 8.

Figure 8 is a chart produced by a computer analysis of the sequence data of the 11873 nucleotide long sequence of Figure 7. Column 1 of Figure 8 gives the arbitrary nucleotide number allotted to the nucleotide of Figure 7. Column 2 apportions the nucleotide number as a fraction of the whole sequence. Column 3 shows the restriction enzymes which will cut the DNA within various short sequences of nucleotides shown in Column 4. The short sequences of Column 4 begin with the nucleotide numbered in Column 1. With the aid of this chart the positions of the restriction sites shown in Figure 6(d) and some of the sequences shown in Figure 6(c) can be determined very accurately. For example sequences II—IV are produced by restriction at the following sites (denoted by the first nucleotide number at the 5′ end of each site).

II	3624—4769
III	6380—7378
IV	10589—11868

Particularly important sites are arrowed in Figure 8. Some of the relevant nucleotide numbers are shown in Figure 6(c), the number given being that of the nucleotide at the 5′ end of each site.

Further sequence analysis of the sub-clones V, VI, VII and VIII shown in Figure 6(c) indicates that the factor IX gene is divided into at least 7 exon regions separated by at least 6 introns. The positions of the exons are shown in Figure 6(a) by the solid blocks labelled t, u, v, w, x, y and z. The "z" exon is much the longest and its 3′-end coincides with the 3′-end of the mRNA. The location of these exons relative to the cDNA sequence is discussed below (section L) and it is clear that the "t" exon shown in Figure 6(a) is not a marker for the 5′-end of the gene, as its sequence fails to match that of the extreme 5′-end of the cDNA clone (see below). This suggests that the factor IX gene will be longer at its 5′-end than the 27 kb region shown in Figure 6, and will contain at least one further exon.

Additionally, pHIX—17 DNA was digested with *Eco*RI. The digested material was resolved on 0.8% agarose gel and a 1.4 kb fragment was isolated in solution by electroelution. It can be stored in the usual manner. This 1.4 kb long molecule was used for the initial sequencing. Only about 1.0 kb is inserted DNA, the remaining 0.4 kb being of pBR322. A 403 nucleotide length of the inserted DNA was sequenced and is identified as O—O′ in Figure 7. The same 1.4 kb fragment was also labelled and used as a probe in Section M.

I. Construction of a vector pAT153/PvuII/8

A derivative of the plasmid pAT153 (Twig & Sherratt, Nature *283*, 216—218, 1980) was prepared for subcloning of *Pvu*II fragments of factor IX genomic clones, and for ease of characterisation of the resultant subclones. Two partially complementary synthetic deoxyoligonucleotides, oligo N3, and, oligo N4, were synthesised by the solid phase phosphotriester method described in Section C above. Each has "overhanging" *Bam*HI and *Hind*III recognition sequences and an internal *Pvu*II recognition sequence. Figure 10 shows the structures of oligo N3 and oligo N4. *Bam*HI and *Hind*III cleave ds DNA to leave sticky or "overhanging" ends. For example *Hind*III cleaves

— AAGCTT
— TTCGAA

between the adenine-carrying nucleotides of each strand leaving the sticky-ended complementary strands:—

— A
— TTCGA

which are present in the oligo N3/N4 combination.

pAT153 was digested with *Hind*III and *Bam*HI and the 3393 nucleotide long linear fragment was separated from the 346 nucleotide shorter fragment by 0.7% agarose gel electrophoresis, followed by electroelution of the appropriate bands visualised by ethidium bromide fluorescence under UV light. After treatment with calf intestinal phosphatase, as described in Section D(i), the *Bam*HI-*Hind*III 3393-long fragment was ligated to an equimolar mixture of oligo N3 and oligo N4 which themselves had been pretreated, as a mixture, with T4 polynucleotide kinase and ATP, to phosphorylate their respective 5′-terminal OH groups. After transforming competent MC 1061 cells (see above) and plating on L-broth plates containing 20 micrograms/ml final concentration of ampicillin, 11 colonies were selected for further analysis. 1 ml plasmid preparation, see Holmes and Quigley, Analytical Biochem. *114*, 193—197 (1981), was isolated from the 11 colonies. The plasmid DNA was then analysed for its ability to be linearised by the restriction enzymes *Bam*HI, *Hind*III and *Pvu*II. Four clones were positive in this assay and one, labelled pAT153/PvuII/8, was selected for sequence analysis by the Maxam-Gilbert method across the newly constructed section of the plasmid. This part of the sequence is shown in Figure 11 along the unique restriction sites. The novel part of the plasmid sequence is underlined: the remainder is present in the parent plasmid pAT153. The vector allows blunt-end cloning (after treatment with phosphatase) into the

inserted *Pvu*II site. The cloned DNA can be excised, assuming that it lacks appropriate internal restriction sites, with *Bam*HI/*Hind*III, *Bam*HI/*Cla*I or *Bam*HI/*Eco*RI double digests. The sites adjacent to the *Pvu*II site are also convenient for end labelling with ^{32}P for characterization of the ends of cloned DNA by the Maxam-Gilbert sequencing method.

J. Sub-cloning of human factor IX gene

The following subcloning experiments were carried out as a first step towards sequencing of the factor IX gene, and to facilitate the isolation of a small DNA fragment to be used as a probe for the analysis of genomic DNA from haemophilia B patients (see sections M).

(i) *Sub-cloning into pBR322 plasmid*

An approximately 11 kilobase *Bgl*II fragment (see Figure 6) within the factor IX DNA insert in lambda HIX—1 clone was inserted into the *Bam*HI site of pBR322. Transformation was carried out in the *E.coli* strain, HB 101. The resulting "sub-clone" was designated pHIX—17 (Figure 12).

(i) *Sub-cloning into pAT153/PvuII/8*

(a) Plasmid DNA from pHIX—17 was prepared and cleaved with *Pvu*II. Five discrete fragments, all derived from the DNA insert of pHIX—17, were isolated. The sizes of these fragments were approximately 2.3, 1.3, 1.2, 1.1 and 1.0 kilobases. These fragments were blunt-end ligated into the *Pvu*II site of the pAT153/PvuII/8 vector and transformed into *E.coli* HB 101. Five clones of recombinant DNA which carried the 2.3, 1.3, 1.2, 1.1 and 1.0 kb fragments were obtained and these were designated pATIXPvu-1, 2, 3, 4 and 5 respectively. Factor IX DNA from pATIXPvu-2 is abbreviated as IV and pATIXPvu-5 as III in Figure 6(c).

(b) Phage DNA from the lambda HIX—1 genomic clone was digested with *Eco*RI. Three different fragments (approximately 5, 2.3, 0.96, kb; see Figure 6), all derived from the insert into the phage, were isolated and inserted in pAT153/PvuII/8 vector at the *Eco*RI site and cloned in *E.coli* HB 101 to form sub-clones. The three resulting clones for each of these fragments were designated pATIXEco-1, 2 and 4 respectively which are shown in the restriction map of Figure 6(d). pATIXEco-1 was further digested with both *Eco*RI and *Bgl*II, and the "overhanging ends" of the restriction sites filled in with deoxynucleotide triphosphates using the Klenow fragment of DNA polymerase I. After isolation of the resulting 1.1 kb fragment by agarose gel electrophoresis and electroelution, it was blunt-end ligated using T4 DNA ligase into the *Pvu*II site of pAT153/PvuII and allowed to transform *E.coli* MC 1061. The resultant sub-clone was designated pATIXBE and the factor IX DNA sequence thereof is abbreviated as II in Figure 6(c).

(c) Phage DNA from lambda HIX—2 was digested with *Hind*III and *Eco*RI giving a 1.8 kb and a 2.6 kb fragment amongst others. These fragments were eluted separately, filled in as described in (b) above, cloned as above into the *Pvu*II site of pAT153/PvuII/8 and allowed to transform *E.coli* MC 1061. The resultant clones were designated pATIXHE—1, and the factor IX DNA sequence thereof is abbreviated as V in Figure 6(c), and pATIXEco—6 and the factor IX DNA sequence thereof is abbreviated as VI in Figure 6(c).

(d) Phage DNA from lambda HIX—3 was digested with *Eco*RI and *Hind* III and the fragments of 2.3 kb and 2.7 kb were sub-cloned exactly as described in (c) above. The resultant clones were designated pATIXEH—1, abbreviation VII in Figure 6(c), and pATIXHE—2, abbreviation VIII in Figure 6(c).

K. Preparation of a library of cDNA clones from human liver mRNA

Messenger RNA was extracted from a human liver and a 20—22 Svedberg unit enriched fraction of mRNA prepared exactly as described for bovine mRNA in Section B above, except that a 'translation assay' was not used. The first steps in the construction of the double-stranded DNA were carried out using the 'Stanford protocol' kindly supplied from Professor P Berg's department at Stanford University, USA. This itself is a modification of Wickens, Buell & Schimke (J.Biol.Chem. *253*, 2483—2495, 1978) and some further modifications, incorporated in the description given below were made in the present work.

For the first strand cDNA synthesis 6 micrograms of poly(A)$^+$ 20—22S human mRNA was incubated with 5 microlitres of 10x buffer (0.5 M Tris-chloride, pH 8.5 at room temperature, 0.4 M KCl, 0.008M $MgCl_2$ and 4 mM dithiothreitol), 20 microlitres of a 2.5 mM mixture of each of the four deoxynucleoside triphosphates, 0.5 microlitres of oligo $dT_{(12-18)}$, 1 microlitre (containing 0.5 microcurie) of [alpha-^{32}P] -dATP, 2 microlitres of reverse transcriptase (14 units per microlitre) and the volume made up to 50 microlitres with deionized water. After incubation for 1 hour at 42°C, the solution was boiled for $1\frac{1}{2}$ minutes and then rapidly cooled on ice. The second strand synthesis was carried out by adding directly to the above solution 20 microlitres of 5x second strand buffer (250 mM Hepes/KOH pH 6.9, 250 mM KCl, 50mM $MgCl_2$), 4 microlitres of a 2.5 mM mixture of each of the four deoxynucleoside triphosphates, 10 microlitres of *E.coli* DNA polymerase I (6 units per microlitre) and making the volume of the solution up to 100 microlitres with deionized water. After incubation for 5 hours at 15°C, S_1 nuclease digestion was carried out by addition of 400 microlitres of S_1 nuclease buffer (0.03 M sodium acetate pH 4.4, 0.25 M NaCl, 1 mM $ZnSO_4$) and 1 microlitre of S_1 nuclease (at 500 units per microlitre). After incubating for 30 minutes at 37°C, 10 microlitres of 0.5M EDTA (pH 8.0) was added. Double stranded DNA was deproteinised by shaking with an equal volume of a phenol: chloroform (1:1) mixture, followed by ether extraction of the aqueous phase and precipitation of ds DNA by addition of 2 volumes of ethanol. After 16 hours at

—20°C, ds DNA was recovered by centrifugation. DNA polymerase I "fill in" of S_1 ends was carried out by a further incubation of the sample dissolved in 25 microlitres of 50 mM tris-chloride, pH 7.5, 10 mM $MgCl_2$, 5 mM dithiothreitol and containing 0.02 mM dNTP and 6 units of DNA polymerase I. After incubating for 10 minutes at room temperature, 5 microlitres of EDTA (0.1 M at pH 7.4) and 3 microlitres of 5% sodium dodecyl sulphate (SDS) were added.

The following part of the protocol differs from the 'Stanford protocol'. The sample was fractionated on a "mini"-Sephacryl S400 column run in a disposable 1 ml pipette in 0.2 M NaCl, 10 mM tris-chloride, pH 7.5 and 1 mM EDTA. The first 70% of the "break-through" peak of radioactivity was pooled (0.4 ml) and deproteinised by shaking with an equal volume of n-butanol:chloroform (1:4). To the aqueous phase was added 1 microgram of yeast RNA (BDH) as carrier followed by 2 volumes of ethanol. After 16 hours at —20°C double stranded DNA was recovered by centrifugation for blunt-end ligation into calf intestinal phosphatase-treated *Pvu*II-cut pAt153/PvuII/8, using T4 DNA ligase (see I and J(ii) above). After performing a trial experiment, it was found that when the bulk of the sample was incubated with 200 nanograms of vector DNA in a suitable buffer (1 mM ATP, 50 mM Tris-chloride, pH 7.4, 10 mM $MgCl_2$ and 12 mM dithiothreitol) and using 10 microlitres of T4 DNA ligase in a total volume of 0.2 ml, then on subsequent transformation of competent *E.coli* MC 1061 cells a total of 58,000 ampicillin-resistant colonies were obtained. Up to 20% of these were estimated to derive from "background" non-recombinants derived by religation of the vector itself. This 20—22S cDNA library was amplified by growing the *E.coli* for a further 6 hours at 37°C. 1 ml aliquots of this amplified library were stored at —20°C in L broth containing 15% glycerol, before screening for factor IX cDNA clones.

L. Isolation and sequence analysis of human factor IX cDNA clones

6000 colonies of the amplified 20—22S human cDNA library were plated on each of ten 15 cm agar plates and after growing overnight were blotted into Whatman 541 filter paper. After preparing filters for hybridisation as described in section E(i) above, the immobilised colonies were probed with a 1.1 kb molecule of [alpha-^{32}P] -nick translated human factor IX genomic DNA isolated from the pATIXBE subclone (Section J, above). This linear 1.1 kb section of factor IX genomic cDNA was isolated from pATIXBE by cleavage with the restriction enzymes *Bam*HI and *Hind*III, followed by separation of the 1.1 kb section from the vector by 1.5% agarose gel electrophoresis. After electroelution, nick-translation was carried out as before and the material used in a hybridisation reaction for 16 hours at 65°C in 3x SSC, 10x Denhardts solution, 0.1% SDS and 50 micrograms/ml sonicated denatured *E.coli* DNA and 100 micrograms/ml of sonicated denatured herring sperm DNA. After hybridisation filters were washed at 65°C successively in 3x SSC, 0.1% SDS (2 changes, half an hour each) and 2x SSC, 0.1% SDS (2 changes, half an hour each). After radioautography, 7 clones were selected as positive, but on dilution followed by re-screening by hybridisation as above, only 5 proved to be positive. Plasmid DNA was isolated from each of these 5 clones and one, designated pATIXcVII, was selected for sequence analysis as it appeared to be the longest of the 5 clones as judged by its electrophoretic mobility on 1% agarose gel electrophoresis. A second shorter clone, designated pATIXcVII was also selected for partial sequence analysis.

Sequencing was carried out by the Maxam-Gilbert method and a 2778 nucleotide long section of sequence is shown in Figure 9. Nucleotides 115—2002 were derived by sequencing clone pATIXcVII. (The actual extent of this clone is greater as it extends in a 5′ direction to nucleotide 17. The sequence between 17 and 111 is inverted with respect to the remainder of the sequence presumably due to a cloning artefact.) Nucleotides 1—130 were derived from clone pATIXcVI which extends from nucleotides 1—1548 of Figure 9. The sequence from Nos. 2002—2778 was derived by isolating 4 additional clones designated pATIX108.1, pATIX108.2, pATIX108.3 and pATIXDB. The first 3 were derived from a mini-library (designated GGB108) of the cDNA clones constructed exactly as described in section K above except that sucrose density gradient centrifugation was used instead of chromatography on "Sephacryl" S—400 to fractionate the double-stranded DNA according to size. A fraction of m.w. from 1 kb—5 kb was selected and an amplified library of 10,000 independent clones containing approximately 20% background non-recombinant clones was obtained. Clone pATIXDB derived from another cDNA library (designated DB1) constructed as described in section K except that total poly A+ · human liver mRNA was used as the starting material and sucrose density gradient centrifugation was used to fractionate the DNA according to size as in the construction of the mini-library GGB108. The complexity of this library was 95,000 with an estimated background of non-recombinants of 50%. Clones pATIX108.1 and pATIX108.2 were selected from a group of 30 hybridization-positive clones isolated by Grunstein-Hogness screening of the mini library GGB108 using a ^{32}P-nick translated probe derived from a *Sau*3AI restriction enzyme fragment, itself derived from nucleotides 1796—2002 of clone pATIXcVII. From pATIX108.1 the sequence of nucleotides 2009—2756 was determined (Figure 9). Following this the sequence of a part of pATIX108.2, specifically nucleotides 1950—2086, provided the overlap with pATIXcVII. The remaining 28 hybridization positive clones were screened by carrying out a triple enzymatic digestion with the restriction enzymes *Eco*RI, *Bam*HI and *Hind*III and screening

the product of the digest for an *Eco*RI restriction fragment extending in the 3' direction from the cut at position 2480. By this approach, clone pATIX108.3 was selected and sequenced from nucleotides 2642—2778. This clone was followed by three A nucleotides, which sequence was confirmed as a vestigial marker for the poly A tail, by the subsequent isolation of clone pATIXDB by a similar method. pATIXDB was sequenced from Nos. 2760—2778 and ended in 42 A nucleotides, thus marking the 3' end of the mRNA.

Figure 9 shows that the predicted amino acid sequence codes codes for a protein of 456 amino acids, but included in this are 41 residues of precursor amino acid sequence preceding the N-terminal tyrosine residue ($\overset{*}{Y}$) of the definitive length factor IX protein. The precursor section of the protein shows a basic amino acid domain (amino acids −1 to −4) as well as the more usual hydrophobic signal peptide domain (amino acids −21 to −36).

The definitive factor IX protein consists of 415 amino acids with 12 potential gamma-carboxyglutamic acid residues at amino acids 7, 8, 15, 17, 20, 21, 26, 27, 30, 33, 36 and 40. Two potential carbohydrate attachment sites occur at amino acid residues 157 and 167. The activation peptide encompasses residues 146—180, which are cut out in the activation of Factor IX (see Background of Invention) by the peptide cleavage of an R—A and R—V bond. This leaves a light chain spanning residues 1—145 and a heavy chain spanning residues 181—415.

The exact location of the boundaries between exons (see Section H, above) and how they are joined in the mRNA is marked in Figure 9. The exons are marked t, u, v, w, x, y, z. It can be seen that there is a rough agreement between the exon domains and the protein regions. For example, the exon for the signal peptide is distinct from that of the GLA region. Also that of the activation peptide is separated from the serine protease domain.

The 3' non-coding region of the mRNA is extensive, consisting of 1390 residues (including the UAAUGA double terminator 1389—1394 but excluding the poly A tail).

The factor IX cDNA is cleavable by the restriction enzyme *Hae*III to give a fragment from nucleotides 133—1440 i.e. a 1307 nucleotide long region of DNA entirely encompassing the definitive factor IX protein sequence. The nucleotide sequence recognised by *Hae*III is GGCC. This fragment should be a suitable starting material for the expression of factor IX protein from suitable promoters in bacterial, yeast of mammalian cells. Another suitable fragment could be derived using the unique *Stu*I site at residue 41 (corresponding to an early part of the hydrophobic signal peptide region) and linking it to a suitable promoter. The nucleotide sequence recognised by *Stu*I is AGGCCT

M. Southern Analysis of normal and patient Christmas disease DNA

(i) *Normal*

The standard (Southern) blotting procedure, Southern, J.Mol. Biol. *98*, 503—517, 1975) was used. In a typical experiment, 10—20 micrograms of human genomic DNA (prepared from uncultured blood cells or cultured lymphocytic cells) were digested with one of a number of restriction endonucleases and loaded onto a single gel slot. Following electrophoresis on 0.8% agarose gel and transfer onto nitrocellulose it was hybridised with a probe of ^{32}P- labelled probe II or of 1.4 kb *Eco*RI fragment (see Section H). Labelling of the probe was carried out by nick translation using the method of Rigby *et al., supra*, modified as follows. About 100 nanograms of the probe was mixed with 40 microcuries of [alpha ^{32}P] dATP (activity about 3,000 Curies/mMole, obtained from Amersham International PLC) in 0.05M Tris-HCl, pH 7.5, 0.01M $MgCl_2$, 0.001M dithiothreitol and dCTP, dGTP, dTTP each at a final concentration of 20 micromolar in a volume of 29 microlitres. To this was added 1 microlitre of "solution X" made up of a mixture of 6 units of DNA polymerase I (*E.coli*), 0.6 nanograms of pancreatic DNase I (Worthington), 1 microgram of crystalline BSA in 10 microlitres of 50% v/v glycerol containing 0.05M Tris-HCl, pH 7.5, 0.01M $MgCl_2$ and 0.001M dithiothreitol. The mixture was incubated for 2 hours at 15°C, after which high molecular weight DNA was purified by chromatography on G—100 "Sephadex". Figure 13 shows the major bands obtained with DNA from normal individuals probed with either probe II (Figure 6) or labelled 1.4 kb *Eco*RI fragment. With each of the 4 enzymes used, *Eco*RI, *Hind*III, *Bgl*II and *Bcl*I, a single major band of about 4.8, 5.2, 11 and 1.7 kb was obtained.

The fact that these restriction fragments had the same length as those observed in the restriction map of clone lambda HIX—1 confirmed that the conditions of Southern blotting were precise enough to detect the factor IX gene in total DNA preparations. This provides the basis for analysis of DNA from the blood of patients with Christmas disease.

(ii) *Christmas patients with gene deletions*

The value of the probes of the invention for the assay of alterations of genes of some patients suffering from Christmas disease has been demostrated as follows. Two patients with severe Christmas disease, who also developed antibodies to factor IX, were selected for study. The DNA from 50 ml of blood was digested separately with *Eco*RI, *Hind*III, *Bgl*II and *Bcl*I and Southern blots prepared for probing with ^{32}P-nick translated probe II (Figure 6). No specific bands were observed with either patient under conditions where a control digest gave the pattern shown in Figure 13. Similarly no bands were observed in

the patients when probe I, III or IV (Figure 6) was substituted for probe II. In order to control for possible mischance of some experimental artefact giving the observed 'negative' signal, a factor IX gene probe (this time pATIXcVII — the cDNA probe) was mixed with an irrelevant autosomal gene probe which was specific for the human AI apolipoprotein (Shoulders and Baralle, Nucl.Acids Res. *10*, 4873—4882, 1982). This experiment showed that patient 1 had normal AI apolipoprotein gene, characterised by a 12 kb band in an *Eco*RI digest, and confirmed that he lacked the 5.5 kb band observed with pATIXcVII and characteristic of the normal factor IX gene. It was concluded that both patients have a sequence of at least 18 kb deleted from their factor IX gene. Two other patients, designated patients 3 and 4, who had also developed antibodies to factor IX gave bands in the normal or abnormal positions on Southern blots with some factor IX gene probes of the invention, but not with others. This suggested that these patients had less extensive deletions of the gene, possibly about 9 kb in length.

These results suggest that diagnosis of haemophiliacs and the heterozygous (carrier) females would be possible in families and this is now under examination. The altered pattern seen in the patient's DNA, whether absence of a band or the presence of a band in an abnormal position, serves as a "disease marker", which can be used to assess for its presence or absence in a suspected carrier. This same test can be applied to antenatal diagnosis, if DNA from foetal cells are available from an amniocentesis. "Genetic diagnosis" should considerably improve existing methods of antenatal diagnosis based on the assay of foetal factor IX protein levels, with the added advantage that the test can be carried out earlier in pregnancy. Genetic methods using natural polymorphisms within the factor IX gene as allelic markers should also make 100% carrier deletion a reality, thereby improving the existing somewhat unsatisfactory methods where probability values are offered to patients.

CLAIMS

1. Recombinant DNA which comprises a cloning vehicle DNA sequence and a DNA sequence foreign to the cloning vehicle, the foreign sequence comprising substantially the following 129-nucleotide sequence (read in rows of 30 across the page):—

ATGTAACATG TAACATTAAG AATGGCAGAT

GCGAGCAGTT TTGTAAAAAT AGTGCTGATA

ACAAGGTGGT TTGCTCCTGT ACTGAGGGAT

ATCGACTTGC AGAAAACCAG AAGTCCTGTG

AACCAGCAG

2. Recombinant DNA which comprises a cloning vehicle DNA sequence and a DNA sequence foreign to the cloning vehicle, the foreign sequence comprising substantially the following 203-nucleotide sequence (read in rows of 30 across the page):—

TGCCATTTCC ATGTGGAAGA GTTTCTGTTT

CACAAACTTC TAAGCTCACC CGTGCTGAGG

CTGTTTTTCC TGATGTGGAC TATGTAAATT

CTACTGAAGC TGAAACCATT TTGGATAACA

TCACTCAAAG CACCCAATCA TTTAATGACT

TCACTCGGGT TGTTGGTGGA GAAGATGCCA

AACCAGGTCA ATTCCCTTGG CAG

3. Recombinant DNA which comprises a cloning vehicle DNA sequence and a sequence foreign to the cloning vehicle, the foreign sequence being substantially the same as a sequence occurring in the human factor IX genome.

4. Recombinant DNA according to Claim 3 wherein the human factor IX sequence has a length of at least 50 nucleotides.

5. Recombinant DNA according to Claim 3 wherein the length of the human factor IX sequence is from 75 to 27,000 nucleotides.

6. Recombinant DNA which comprises a cloning vehicle sequence and a DNA sequence foreign to the cloning vehicle, wherein the foreign sequence includes substantially the whole of an exon sequence of the human factor IX genome.

7. Recombinant DNA which comprises a cloning vehicle sequence and a DNA sequence foreign to the cloning vehicle, wherein the foreign sequence comprises a DNA sequence which is complementary to the human factor IX mRNA.

8. Recombinant DNA according to Claim 3, 4 or 5, wherein the cloning vehicle is a modified pAT153 plasmid prepared by ligating a *Bam*HI and *Hind*III double digest of pAT153 to a pair of complementary double sticky-ended oligonucleotides having a DNA sequence providing a *Bam*HI restriction residue at one end, a *Hind*III restriction residue at the other end and a *Pvu*II restriction site in between.

9. Recombinant DNA according to Claim 8 wherein the pair of complementary oligonucleotides are of formula:—

5′ GATCCAGCTGA 3′

. . . .

. . . .

3′ GTCGACTTCGA 5′

10. Recombinant DNA which comprises a cloning vehicle sequence and a DNA sequence

foreign thereto which hybridises to a 247 base-pair sequence of bovine factor IX DNA complementary to messenger RNA and indicated in Figure 5 by the arrows at each end thereof.

11. A host transformed with at least one molecule per cell of recombinant DNA claimed in any preceding claim.

12. A host according to Claim 11 in the form of *E.coli.*

13. A host according to Claim 11 in the form of mammalian tissue cells.

14. A process of preparing a host transformed with recombinant DNA as claimed in any one of Claims 1 to 7, which process comprises:—

(1) synthesising an oligodeoxynucleotide probe having a nucleotide sequence comprising that occurring in bovine factor IX messenger RNA coding for amino acids 70—75 or 348—352 of bovine factor IX and labelling the oligodeoxynucleotide to form a probe;

(2) preparing complementary DNA to a mixture of bovine RNA;

(3) inserting the complementary DNA in a cloning vehicle to form a mixture of recombinant bovine cDNAs;

(4) transforming a host with said mixture of recombinant bovine cDNAs to form a library of clones and multiplying said clones;

(5) probing the clones with the synthetic oligodeoxynucleotide probe obtained in step 1 and isolating a resultant recombinant bovine factor IX cDNA-containing clone;

(6) digesting the recombinant bovine factor IX cDNA from said clone with one or more enzymes to produce a bovine factor IX cDNA molecule containing a shorter sequence of bovine factor IX DNA; and

(7) probing a library of recombinant human genomic DNA in a transformed host with the shorter sequence bovine factor IX cDNA molecule, to hybridise the human genomic DNA to the said recombinant bovine factor IX DNA and isolating the resultant recombinant DNA-transformed host.

15. A process of preparing a host transformed with recombinant DNA as claimed in Claim 1, 2 or 7, which process comprises probing a library of clones containing recombinant DNA complementary to human mRNA with a probe comprising a labelled DNA comprising a sequence complementary to part or all of an exon region of the human factor IX genome.

16. A DNA molecule comprising an at least 15 nucleotide long sequence of part or all of substantially the 129-nucleotide sequence set forth in Claim 1.

17. A DNA molecule comprising an at least 15 nucleotide long sequence of part or all of substantially the 203-nucleotide sequence set forth in Claim 2.

18 A DNA molecule comprising an at least 15 nucleotide long sequence of part only of the DNA sequence of the human factor IX genome.

19. A DNA molecule comprising a sequence of length at least 15 nucleotides substantially the same as a sequence complementary to part or all of that occurring in human factor IX mRNA.

20. A DNA molecule according to any one of Claims 16 to 19 of length at least 50 nucleotides.

21. An artificial DNA molecule comprising a sequence substantially the same as a sequence of length at least 15 nucleotides occurring in the human factor IX genome.

22. An artificial DNA molecule according to Claim 21 comprising substantially only exon sequences.

23. A labelled diagnostic probe comprising a DNA molecule having a single-stranded or double-stranded probe sequence of from 15 to 10,000 nucleotides long of DNA sequence defined in Claim 16, 17, 18 or 19 or its complementary sequence.

24. A probe according to Claim 23 having a probe sequence from 20 to 5,000 nucleotides long.

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